Pollination ecology and molecular systematics of Diuris (Orchidaceae)

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21st April, 2010

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Abstract

The Australian terrestrial orchid genus *Diuris* is currently recognised to contain at least 61 species, with numerous new taxa expected to be recognised in the near future. Species are restricted to Australia, with the exception of *Diuris fryana*, which is endemic to Timor. Species of *Diuris* are well represented in the southern parts of western and eastern Australia, separated by the Nullarbor Plain, with a few species found in tropical Queensland. The eastern and western species mostly fall into morphologically distinct groups suggestive of distinct phylogenetic lineages.

Despite considerable variation between and even within species, *Diuris* species share certain important features. Most species occur in open forest and woodland and have flowers that bear a resemblance to Australian native 'egg and bacon' pea flowers of the tribes Bossiaeae and Mirbeliae, with which they are frequently sympatric. In some species, the resemblance is very close, in others it is more general. Most existing work on pollination in this taxon is of an anecdotal nature, with only one formal study prior to this project, of one species (*Diuris maculata* at Fern Tree Gully, near Melbourne, Victoria in 1986). The Beardsell *et al.* (1986) study proposed that this orchid was a non-rewarding floral mimic of pea flowers of the genera *Daviesia, Pultenaea* and *Dillwynia*. It was sympatric with the peas, with which it bore a visual resemblance, flowered at the same time and was visited by native bees, plus a wasp species that pollinated the pea flowers.

The overall purpose of my project was to advance knowledge of the pollination biology of *Diuris*. In particular, I planned to (i) test the effectiveness of AFLP markers for identifying the source of pollinium remnants collected from the bodies of putative

pollinators, (ii) conduct detailed pollination studies on two species in the Sydney region (*D. maculata* and *D. alba*) to test the generality of the conclusion of floral mimicry in *Diuris,* drawn from the 1986 study in Melbourne; (iii) survey pollinator interactions in a range of taxa (*D. aurea*, *D. punctata* and *D.* sp. aff. *punctata* (Mellong Swamp)), using pollination observations, imaging of ultraviolet visual cues, colorimetric analysis of putative model and mimic flowers, testing for nectar production and DNA-based identification of pollinaria removed from captured insects, to test for patterns in pollinator-plant associations, and (iv) place these observations in the context of a complete a phylogenetic analysis of the genus *Diuris.*

These main experimental findings are summarised as follows:

1. Amplified Fragment Length Polymorphism (AFLP) was shown not only to be capable of distinguishing many species, but also to possess high sensitivity. This latter feature had not been exploited previously. (See Chapter 2)
2. The pollination mechanism of *Diuris maculata* in this population was shown to be similar to the original Beardsell *et al.* (1986) study, but there were some significant differences – the timing of orchid flowering early in the flowering season of putative model pea species (cf. synchronised flowering in the Victorian population) and the role of male bees (cf. various male and female bees) in pollination, which may be quite common in *Diuris*. (See Chapter 3)
3. *Diuris alba* has flowers that are similar in form, but not colour, to other, putative pea-mimicking *Diuris* species. *Diuris alba* at Munmorah, New South Wales, was found to occur in a variety of

habitats including sites where pea flowers are absent or rare and the pollination success was found to be not dependent on pea flowers. This species was also found to produce a meagre nectar reward. (See Chapter 4)

4. Unpublished pollination data were obtained for a number of *Diuris* taxa (*Diuris aurea* (Castlereagh Nature Reserve), *D. punctata* (Castlereagh Nature Reserve), *D.* sp. aff. *punctata* (Mellong Swamp), *D. arenaria* (Tomaree National Park) and *Diuris sulphurea* form Stringy Bark Ridge, Pennant Hills. It was found that *D. aurea*, *D. punctata* and *D.* sp. aff. *punctata* showed pollination features consistent with Batesian-type floral mimicry of yellow 'egg and bacon' pea flowers, despite the latter two taxa having a white floral anthoxanthin base colour (with pink/purple suffusions). Additionally, preliminary data was obtained for *D. aurea* and *D.* sp. aff. *punctata* that showed higher pollination success for plants clustered some distance from yellow pea flowers than was obtained for plants scattered among yellow pea flowers. The taxon *D. arenaria* was shown to have higher reproductive success, when scattered amongst yellow 'egg and bacon' pea flowers than would be expected for a Batesian-type mimic, a result suggestive of a more generalised pollination strategy. Meagre nectar was found in one plant of *Diuris sulphurea* tested for nectar production, an interesting result that requires confirmation with further testing.

5. The molecular phylogenetic analysis of *Diuris* (Orchidaceae) based on AFLP and ITS (Internal Transcribed Spacers of Ribosomal DNA)

revealed three major clades and a basal species. *Diuris sulphurea* (subg. *Paradiuris*) is shown to a monotypic sister group to the rest of *Diuris*. (See Chapter 5)

The conclusions of the study can be summarised as follows:

1. Some species show close visual mimicry of specific model species (strict Batesian mimicry) e.g. *Diuris aequalis* is proposed to mimic *Gompholobium* spp.
2. Many species show a more generalised mimicry of peas (loose Batesian-type mimicry) e.g. *Diuris maculata* shows general similarity to 'egg and bacon' peas. Over its wide distribution (southern Victoria to north of Sydney, New South Wales) this orchid occurs at many sites and its pollination is likely to involve dozens of species of both peas and pollinators.
3. Some species show apparent dependence on mimicry while showing only aspects of similarity, with other different, perhaps flamboyant features suggestive of non-model mimicry e.g *Diuris* sp. aff. *punctata* (Mellong Swamp). This species shows pollination outcomes in the presence of the yellow pea *Dillwynia glaberrima* as expected for a yellow-flowered pea mimic. Its flowers show general pea-like form. However, the pink/purple floral colouration is quite different to the sympatric yellow peas. It is also noticeably fragrant. It is suggested that the flowers of this species are 'exciting' to a bee foraging on pea flowers because its flowers possess strong floral cues, which could be considered to transcend strict mimicry.

4. Generalised pollination e.g. *Diuris alba* from Munmorah, New South Wales. In this case flowers were shown to have pea-like floral form, but with different colour (white cf. yellow), fragrance and nectar. Plants were shown to have high pollination success both in the presence and absence of pea flowers. The pollination system was analysed using colorimetric analysis with a model of predicted colour-based foraging errors. This pollination system was proposed to have evolved from pea mimicry and not entirely disconnected from it. Aspects of Batesian-type mimicry, non-model mimicry, the 'magnet effect' and the presence of a meagre nectar reward may all contribute to high pollination success in varied environments.
5. *Diuris sulphurea* is the most widespread of all *Diuris* species in eastern Australia, forms a basal clade to the rest of the genus and may show ancestral pollination features. Such a widespread species is unlikely to mimic a single pea species and must show fairly generic pea mimicry. It has a colony-forming growth habit, produces nectar and has high reproductive success. An understanding of its pollination mechanism is likely to lead to insights of pollination evolution within the genus.

The phylogenetic data allow the following interpretations of current patterns of *Diuris* pollination:

1. The finding that *D. sulphurea* forms basal clade to all other *Diuris* suggests that this species may possess ancestral features, including pollination mechanism, which on the basis on preliminary evidence

would appear to be fairly generalised (guild) pea mimicry combined with nectar reward. Its pollination mechanism, combined with a colony-forming habit might be expected to promote significant self-pollination.

2. Knowledge of species groupings (clades) will aid in focusing pollination studies since clades can be expected to contain species sharing morphological and pollination features.

3. Yellow base colour can be inferred to be ancestral in *Diuris*, with pink/purple colouration being a synapomorphy in *Diuris* subg. *Diuris* sect. *Purpureo-albae* (plus some species within *Diuris* subg. *Xanthodiuris*, sect. *Pedunculatae*, e.g. *D. venosa*). Preliminary data suggest that despite the floral colour difference, species closely related to *D. punctata* appear to mimic yellow pea flowers and have a similar pollination mechanism, while another closely related species, *D. alba* has been shown to have generalised pollination. Therefore, a detailed understanding of pollination of the species *D. aurea*, *D. punctata*, and *D. alba* can be expected to provide considerable information about pollination within this large species group and also to provide important insights into the evolution of Batesian-type mimicry in this orchid group.

I propose the following hypothesis about the role of Batesian-type mimicy (which includes strict Batesian mimicry as commonly understood and looser forms, such as mimicry of a guild of Mullerian mimics) in the pollination systems of

Australian east coast *Diuris* species. Strict Batesian mimicry is highly specialised and inevitably leads to rarity, and there are consequently few examples. However, the looser type of Batesian mimicry (sometimes termed 'guild mimicry') exemplified by *Diuris maculata* permits the exploitation of many ecologically similar environments, in which basically similar, but distinct pea flower species may all serve as models for this orchid. Within *Diuris*, subg. *Diuris*, sect. *Purpureo-albae* there are numerous species with pink/purple, or white base colour. Many of these, paradoxically, appear to depend on association with yellow 'egg and bacon' peas for pollination. Phylogenetic evidence suggests that these species have undergone recent and rapid evolutionary radiation. This could be viewed as a shift toward even looser Batesian-type mimicry and could account for their evolutionary success. *Diuris alba* represents a group of species which have developed a sufficiently generalised pollination system to be independent of pea flowers, although high reproductive success in the presence of pea flowers would suggest that the link to pea mimicry is not completely broken. It thus may be reasonably termed a 'non-model' mimic and it likely benefits from the 'magnet effect' of being in the proximity of abundant rewarding species (which often happen to be pea flowers). As resemblance of this species to pea flowers is somewhat unclear, it may not be meaningful to view pea flowers as 'model' flowers, or indeed this orchid as a pea 'mimic'.

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Acknowledgements relating to each of the published research chapters can be found at the end of each of these chapters as they appeared in the published articles.

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Chapter 1

General Introduction

1. A Brief History of Ideas in Pollination Biology

Modern ideas about sexuality in plants, mechanisms of pollination and its significance in maintaining genetic diversity, the capacity of species to adapt to changing conditions, and for new species to evolve may seem perfectly reasonable to a 21st century biologist, but as is the case with so much of science, such knowledge is part of a long story with many twists and turns, and occasional major conceptual or theoretical advances. So many people have contributed to the present day knowledge of pollination biology that even a summary of their contributions would be beyond the scope of this brief introduction. This subject has been ably reviewed in detail elsewhere (Baker 1983; Proctor *et al.* 1996). This introduction focuses on the key ideas in pollination biology in order to attempt an understanding of how we have arrived at current knowledge and what questions remain in need of study.

Theophrastus (4th century B.C.) referred to a famous Mesopotamian bas relief (of the 9th century B.C.) illustrating manual pollination of date palms by shaking inflorescences from a staminate palm over a pistillate palm (Baker 1983) and he referred to the pollination in terms of male and female contributions. Such an observation is suggestive of an enlightened awareness of plant sexuality in plants from ancient times. Ancient ideas do not seem to equate with modern ideas of plant sexuality and many centuries were to pass before the idea of sex in plants became well established. A number of botanists towards the end of the 17th century, such as Nehemiah Grew, suggested a role of stamens and pollen in seed formation. Rudolph

Jacob Camerarius, who was a botanist and physician at Tubingen in Germany, performed a number of important early experiments on floral function. For example, when anthers were experimentally removed from flowers of the castor oil plant *Ricinus communis*, the female flowers failed to set seed. Carulus Linnaeus put forward his 'sexual system' of seed plant classification in 1735 (Baker 1983). He recognized that male and female floral parts were constant within taxa, but this did not imply a true understanding of the function of these parts. Evidence accumulated by numerous botanists led to the idea of sexuality, in terms of the existence of functional male and female organs in plants, being well established by about 1750.

Aristotle observed flower constancy by bees. However, the role of insects in floral visitation was not generally appreciated and for centuries they were presumed to be thieves of flowers rather than performing a service (Proctor *et al.* 1996). In 1750, Arthur Dobbs observed bees around his home in Carrickfergus in County Antrim, Ireland, and reported to the Royal Society how bees would visit flowers of a certain species in a meadow, flying past flowers of many other kinds in doing so. The pollen, then known as 'Farina' was noted to be collected and the suggestion was made that bees were instrumental in promoting the 'Increase of Vegetables'. This was followed by important work by Joseph Gottlieb Kolreuter, Professor of Natural History at the University of Karlsruhe, who performed numerous systematic experiments establishing the importance of insects in the pollination of many plant groups. The idea of cross pollination would have been difficult to understand at this time, since most flowers are hermaphroditic (possessing both male and female organs), an arrangement that most botanists would assume could only result in self-pollination.

Christian Konrad Sprengel, widely considered to be the father of pollination biology, made numerous perceptive observations and deductions concerning floral colour, nectar guides and nectaries and the role of floral rewards in insect pollination of flowers. These were published in his classic book of 1793 'Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen' (The revealed secret of Nature in the structure and fertilization of flowers) (Sprengel 1793). Sprengel was also aware of floral adaptations for cross pollination such as protandry (maturation of male organs in hermaphroditic flowers before the female organs) and went on to state "It seems that nature is unwilling that any flower should be fertilized by its own pollen" (Baker 1983). The ideas proposed in this book were generally ridiculed and made little impact for half a century. Although the ideas of Sprengel showed great insight, it appears he did not grasp the significance of how cross-pollination could be of benefit to plants. In the absence of this connection his ideas must have appeared obscure - as was suggested many years later by the great 19th century anthecologist Hermann Muller (Muller 1883 in Baker 1983). However, as Proctor, Yeo and Lack (1996) point out, Sprengel's ideas seem to have been quite widely known and discussed, at least among a small number of leading scientists.

Sprengel discovered that certain flowers with nectar guides lacked nectar, such as the terrestrial orchids *Orchis latifolia* and *Orchis morio* (now *Anacamptis morio*). This initially caused him great concern as his theory of flowers could be called into doubt. Further observations, however, convinced him that certain flowers such as those of *Aristolochia clematitis* were indeed 'false nectar flowers' (Sprengel 1793; see also

Vogel 1996. This book includes an English translation of the Introduction to Sprengel's book by Peter Haase).

The modern concept of sexuality in plants was not understood until the 19th century. Prior to this time it was presumed that that the pollen contained the 'seed' of the plant, which after implantation on the stigma, developed in the ovule (Amici, cited in Baker 1983). This made the female parts (or plant, in the case of dioecious plants) merely a receptacle for the seed, an idea comparable to the 'humunculus' theory of human and animal sexuality. John Smith, at Kew Gardens (Smith in Baker 1983) made the crucial observation in 1841 that *Alchornea ilicifolia* seeds could develop in pistillate plants, with no sign of an androecium. In doing so he destroyed the idea that 'every grain of pollen furnishes a germ' and demonstrated apomixis at the same time.

Charles Darwin was greatly influenced by Sprengel, and in addition to publishing the famous book 'The Origin of Species' (Darwin 1859) also published major works in pollination biology, including his major treatise on orchid pollination (Darwin 1877). Like Sprengel, Darwin appreciated the role of cross-pollination and stated "Nature tells us in the most emphatic manner that she abhors perpetual self-fertilisation". Darwin's great contribution was to be able to take the next logical step from Sprengel in not only showing the prevalence of outcrossing mechanisms in plants, but also to provide an explanation of its importance – as a means of facilitating natural selection of beneficial characters. It is interesting that Darwin did not accept Sprengel's ideas of deceptive orchids: 'we can hardly accept so gigantic an imposture' (Darwin 1862, (also 1877), cited in Nilsson 1980). Later work by Delpino (1873-1874)

supported Sprengel's ideas on deceptive pollination and finally, work by Daumann (1941) disproved Darwin's assertion, (see Nilsson 1980).

Darwin's work was hugely influential and inspired many scientists to work in this field. A vast literature had developed in descriptive pollination studies by the end of the 19th century (see Knuth 1909). Baker (1983) described the state of knowledge to this point as the 'old testament' in pollination biology, a biblical analogy to the edifice of knowledge that had been achieved and representing a kind of climax before the next great leap in knowledge. As Proctor *et al.* (1996) observed, the beginning of the 20th century saw a decline in these classical studies of pollination for various reasons, one being that they had run out of momentum. This was due in part to a basic limitation of Darwin's evolutionary model - the absence of a mechanism to explain inheritance (Wyhe 2003).

Experimental sciences increased in importance in the early 20th century, particularly the new science of genetics after the classic breeding experiments in garden peas of the Austrian monk Gregor Mendel were rediscovered (Henig 2000). The discovery of the role of DNA (deoxyribose nucleic acid) as the active agent in inheritance and the development of population genetics has reignited interest in pollination studies, especially in the modern era of molecular biology (Watson and Crick 1953).

2. Flower Visits by Hymenopteran Insects

Flowers are pollinated by a number of major insect groups, including the beetles (Coleoptera), moths and butterflies (Lepidoptera) and flies (Diptera). But it is the

Hymenoptera (bees, ants and wasps), and particularly the bees that is of greatest importance. Various sorts of wasps visit flowers and play some role in pollination. Wasps typically feed their young captured prey. Adults frequently visit flowers for nectar. Bees are typically viewed as a separate group to the wasps, although in reality they are derived from sphecid wasps (O'Toole and Raw 1992). The major difference is behavioural as almost all bees are entirely vegetarian with pollen being the main protein source for the developing young.

The vast majority of flowers pollinated by an animal vector are visited by bees (Kevan and Baker 1983; Michener 2000). Flowers may possess features for generalised pollination. These may be pollinated by bees, but other insect groups, such as beetles and flies.However, many flowers are specialised for bee pollination and possess features that only permit access to a reward by bees, which are able to read the foraging cues of the flowers (Sprengel 1793) and possess the behavioural adaptations required to exploit these flowers. Specialised pollination by flies, birds, bats or mammals is also significant, but these are in the minority compared to those pollinated by bees.

2.1 Nectar guides

Christian Konrad Sprengel's major contribution to pollination biology was as an amateur naturalist and perceptive observer and, in particular, he developed the "nectar guide theory" of visual cues in flowers leading pollinating insects to hidden sources of nectar and pollen (Barth 1991; Vogel 1996; Proctor, Yeo and Lack 1996). Sprengel recognized the importance of overall flower colour, noting that insect pollinated flowers tend to have bright colours which contrast with their surroundings. He was also aware of the significance of fragrance as a floral attractant, and visual cues (nectar guides)

leading to the nectar contained within nectaries. Experimental confirmation of Sprengel's ideas had to wait until the early 20th century with the pioneering work of the German scientists Hans Kugler and Karl Daumer, who showed that nectar guides occur in various forms (see Barth 1991, Chapter 15):

Rings: An example of this form of nectar guide is the forget-me-not (*Myosotis* spp.). These have small radially-symmetric flowers of soft blue colour. The colour contrast is provided by a yellow ring at the flower centre.

Corona: Barth gives the example of *Narcissus poeticus*, which has a corona of yellow and red in contrast to the white perianth.

Colour patches. Numerous species have flowers with a prominent spot. The orchid *Dendrobium infundibulum* is white in floral base colour and has a prominent yellow patch on the labellum. It is thought that this species, along with *Cymbidium insigne*, is a Batesian mimic of *Rhododendron lyi* (Kjellson *et al.* 1985).

Spots. In other cases, flowers such as foxglove (*Digitalis* spp.) have rows of spots leading to the flower centre.

Stripes. Many flowers have a number of stripes, or lines pointing to the flower centre, such as the heartsease pansy (*Viola tricolor*).

Flowers are often multi-coloured with the colour-contrast forming a guide to the food source. The floral symmetry of nectar guides has been found to conform with the

overall floral symmetry (i.e. radial vs. bilateral symmetry) (Sprengel 1793: Manning 1956; Dafni and Kevan 1996). These colour patterns are often known as nectar guides since they are involved in the orientation of pollinators to nectar rewards (Kevan and Backhaus 1998). There seems to be a general rule for colour combinations of shorter wavelengths at the flower periphery and enrichment of longer wavelengths in a central region (Lunau 1992; Heuschen *et al.* 2005). Many examples can be found, such as Shasta daisy (*Chrysanthemum maximum*). These daisy "flowers" (really a head, or capitulum, of many modified flowers) have white florets with a yellow center, and like most white flowers, lack a UV colour component. Thus colours reflected that are visible to a bee (with poor red vision) include blue + green spectral components for the outer parts, but green only for the centre. Heuschen *et al.* (2005) found that the inner colour of many radially symmetrical as well as many zygomorphic flowers appears to bees to be very similar to that of pollen, suggesting that the nectar guides of many flowers show a generalised form of pollen mimicry.

2.2 Insect Perception of Colour

Sprengel (1793) was convinced from his field observations that insects possessed colour vision, but did not conduct any experimental tests of this hypothesis. Early experiments into insect colour vision are reviewed by Barth (1991). In the early 20th century, the idea of insect colour vision was controversial. Karl von Frisch in 1914 performed experiments with bees trained to feed at a checkerboard of squares of shades of gray plus a blue square. After training, bees would fly to the blue square even when moved around and food reward was no longer offered. They could not be trained to visit gray squares, or black and white ones. It turns out that bees cannot respond to variation in brightness (Backhaus 1992). About a decade later Alfred Kuhn and Robert Pohl (see

Barth 1991) performed training experiments using very well defined wavelengths – either lines in a mercury spectrum or parts of a continuous spectrum, to show that the bee can see a colour spectrum about as broad as human vision, but shifted toward shorter wavelengths.

Human vision is well studied and has been shown to be trichromatic, that is to involve the spectral sensitivities of red, green and blue sensitive colour receptors in the retina of the eye (see Dyer 1996). Research into insect vision has shown that hymenopteran insects also possess a trichromatic colour vision system, but shifted towards the ultraviolet and based on ultraviolet, blue and green colour receptors (Kevan and Backhaus 1998). There is a pervasive view that bees lack red colour vision, but this would seem to be overstated. Certainly, bee perception of red colours is poorer than in humans, but evidence from the step functions of floral colours and colour receptor responses in bees would suggest that red colour sensitivity in bees would extend to about 650 nm (Chittka and Waser 1997).

2.3 Visual Cues in Flowers

The fact that many insects possess ultraviolet vision and that flowers frequently possess ultraviolet visual cues has led some researchers to the view that there is some sort of mysterious and secret communication between insects and flowers (Chittka *et al.* 1994). Ultraviolet vision is in fact common and widespread among insect groups and vertebtrates, but is lacking in humans (Kevan *et al.* 2001). Kevan et al. (2001) review various aspects of ultraviolet in relation to hymenopteran insects and their foraging behaviour. They pointed out that in Pre-Cambrian times, before forms of oxygen became a component of the atmosphere, ultraviolet levels must have been much higher

than today. Receptors for ultraviolet in bees are more sensitive than the green and blue receptors, but this may be a simple consequence of the fact that ultraviolet is highly attenuated in the daylight spectrum (Kevan 1972; Kevan *et al.* 2001).

Evolutionary studies suggest that trichromatic colour vision, including ultraviolet sensitivity, is phylogenetically ancient in insects and crustaceans (Chittka 1996). It is interesting to consider whether the vision of insects, particularly hymenopterans, of which bees are the most notable floral visitors, evolved under the selection of floral colours, or vice versa. There is strong evidence that the vision of insects was pre-adapted (meaning independently previously evolved) to discriminate floral colours as trichromatic insect vision is likely evolutionarily ancient and would have pre-dated floral evolution. Therefore, flowers would seem to have adapted to this capability of insects to see colours, including ultraviolet.

Daumer (1958) used photographic methods to study floral colours as perceived by bees. Flowers were photographed using filters with transmission bands corresponding to the three primary colours of bee vision. He was able to describe flower colours in terms of bee vision. Chittka *et al.* (1994) used reflectance spectrophotometry to more precisely survey floral colour in the insect visual spectrum, from 300 to 700 nm wavelength (ultraviolet through to red), of flowers of 1063 plant species, and included foliage measurements for reference. Just 10 distinct types of reflectance curves were found, which show clear step functions i.e. combinations of presence or absence of reflectance of colour within an ultraviolet, blue, green, or red wavelength bandwidth. For example, one colour class of flowers, which appear yellow to humans, and which was found in 16.6% of measurements, comprises absence of reflectance from 300 to about 520 nm,

after which a sudden step of reflectance is found, through the green range, and continuing in the red end of the spectrum. Another class of flowers, which appear yellow to human eyes was found to possess an ultraviolet bandwidth, lacked blue reflectance, and reflected again from about 520 nm and into the red range. This was found in 13.1% of measurements. Flowers which appear white to human eyes lacked ultraviolet reflectance, but possessed reflectance from 400 nm continuously into the red end of the spectrum (i.e. had blue + green + red colour components). This was found in 19.7% of measurements. Flowers reflecting in the ultraviolet and red bandwidths only are very rare (1.6% of measurements) and appear red to human eyes, but essentially ultraviolet only to bees. An example is the red poppy, *Papaver rhoeas*.

The studies of Kevan (1972) and Chittka *et a*l. (1994) make it clear that it is not appropriate to attempt to compare floral colours in accordance with human perception and that comparisons can only meaningfully be made by methods such as reflectance spectrophotometry, where the full spectral sensitivity of insect vision is taken into account. Ultraviolet visual cues are given special treatment in this research project, not because ultraviolet is particularly special as a colour, but because ultraviolet visual cues are known to be important in Australian "egg and bacon" peas of tribes Mirbeliae and Bossaiaeeae (Kay 1987). Analogues of the pea flower colour contrast were subsequently shown to be present in *Diuris* orchid flowers, as would be expected if these orchids can be considered mimics of pea flowers (Indsto *et al.* 2006; Indsto *et al.* 2007).

2.4 The Role of Floral Form

In addition to varying colours, flowers differ markedly in basic shape and size and other visual cues. This was not examined in the context of floral mimicry in this project as no

suitable method for analysis could be determined. Despite this, I believe this is an important floral feature and so will be given an introductory treatment here. Barth (1991) discussed pioneering work of Karl von Frisch who surprisingly found that bees could not distinguish a range of filled (solid) shapes such as a circle, square or triangle. He found bees can respond to "contour length", or figural intensity, which is the "edginess" of a shape. Star shapes, with increasing numbers of points would represent increased figural intensity, as do concentric circles, with increasing numbers of circles being increased figural intensity. Figural quality is another property detectable by bees and refers to the nature of a shape e.g. a circle, square or a triangle comprised of dots represent progressively increased figural quality.

Manning (1956) worked with bumblebees to study visitation behaviour. Bumblebees are large and tend to cover much of the flowers they visit. For this reason Manning used large flower models 12-15 cm of blue colour and used yellow marks to simulate nectar guides. He found the bumblebees were initially attracted to the edges of the model flowers and fly straight to the model from a distance of ~60 cm. The same thing can be observed with real large flowers, such as poppies (*Papaver* spp.). The bumblebees fly to models with and without nectar marks with equal frequency, but hover over models longer with guide marks. Petaloid, actinomorphic (i.e. with star-shape and radial symmetry) models without guide marks were also used. Bumblebees still were more attracted to the edges, but landed more frequently at the centre than they did with similar models less deeply cut. When the petaloid models had a honey guide consisting of yellow lines running down the centre of each "petal" the reactions to the centre increased with more visits to the centre than the edge recorded. Scent was noted to be a stronger influence on bee alighting behaviour than its colour pattern. Free (1970)

performed a similar study with honeybees, but with yellow model flowers about 30 mm in diameter, using blue nectar guides. He found that the honeybees preferred radially symmetric over bilaterally symmetric models and models with a disruptive outline (i.e. shapes such as stars, or lobed circles) to circular models. The honeybees landed more often on models with nectar guides than plain models. Dotted lines were shown to be more attractive than solid lines and a group of dots were more attractive than a ring. Leppick (1953), found that bumblebees showed a strong preference for bilaterally symmetric flowers over those with radial symmetry, in contrast to the above finding for honeybees.

Neal *et al.* (1998) described evolutionary patterns in floral form and symmetry. They considered the primitive state of perianth organs to be (a) a spiral (helical) arrangement of organ members, (b) an indefinite number of each floral organ (i.e. many petals or stamens) and (c) similar morphology of all members of each organ type. Such primitive flowers can be considered asymmetric since there is no repetition of pattern. However, this is an active area of research and no clear consensus on these patterns of floral evolution has yet been achieved (Endress and Doyle 2006). Neal *et al.* (1998) considered the derived state for flowers to be (a) a whorled arrangement of floral organs, (b) a definite number of each floral organ and (c) dissimilar morphology among the members of an organ type. A good example of a highly derived flower type then would be a pea flower with 5-lobed calyx and 4-partite corolla whorls, and specialisation of corolla members into banner, keel and wings of the flower. It would also follow that zygomorphic (bilaterally symmetric) flowers are best capable of showing these derived features.

3. The Antiquity of the Association of Bees and Flowers – Linked in Evolutionary Stasis

Flowering plants, according to the fossil record, appear to have undergone two major periods of rapid evolutionary diversification (Proctor *et al.* 1996, Chapter 14). Flowering plants may have appeared about 150 million years ago and to have completed a major phase of evolution by the middle of the Cretaceous. By the end of the second major phase of evolution from about 75 to 50 million years ago, the majority of the living plant families had appeared. Numerous examples also exist of fossils from this period show a close resemblance in form to plants living today. Molecular data offer highly divergent timings for the origin of angiosperms, although a Middle-Jurassic to early-Cretaceous origin, pre-dating the fossil record appears likely (Bell *et al.* 2007).

The evolution of flowering plants was linked to the evolution of their insect pollinators, particularly bees, and evidence exists that bees have a similar antiquity. An advanced stingless bee, named *Trigona prisca*, a representative of the important bee family, the Apidae, has been found preserved in Cretaceous amber between 74 and 96 million years old (Michener and Grimaldi 1988). Remarkably, this bee shows a high similarity to species in existence today and suggests not only that social behaviour in bees is very ancient, but that these bees have shown considerable evolutionary stasis in morphology since this time. Naturally, this bee has attracted much interest. A recent study of this bee has resulted in a revision of the name: it is now placed in a new genus, *Cretotrigona* and its age revised to 65-70 million years (Engel 2000). Thus, it appears that following the appearance of flowers and bees, constraining selective pressures have been important in maintaining the form of certain lineages of bees and flowers to the present day. An even older bee (*Melittosphex burmensis*), approximately 100 million

years old has recently been discovered (Poinar and Danforth 2006). This further reinforces evidence of linked evolution of flowers and pollinating bees to earliest times. The link between a pollinator and the flower(s) it visits would result in floral form being constrained for optimal perception by a pollinator, which in turn must retain the adaptive biological features that enable it to locate and feed on the flower (pollination being an incidental by-product of visitation from the perspective of a floral visitor). The example of *Trigona* (*Cretotrigona*) *prisca* would seem to be a classic case of punctuated equilibrium in evolution, where rapid evolutionary change in response to environmental change or innovation, settles into stasis as constraining forces retain favourable adaptive features (Gould and Eldredge 1972).

4. Specialised Features of Bee-pollinated Flowers

Floral features that are adaptive for bee pollination must have been fundamental in the evolution of many flowering plant families with the result that many of these floral features are shared among diverse flowering plant lineages. The zygomorphic floral form is important here and is represented in numerous plant families, including those families of particular importance in this study, the Fabaceae and Orchidaceae. Zygomorphy may have arisen repeatedly as a class of adaptations to bee pollination. In contrast, dense head-like inflorescences, especially those of composites (Asteraceae) tend to be visited by a diversity of pollinators, including bees.

Two basic variants of zygomorphic flowers have been recognized in the pollination literature. In the sternotribic form, the stamens and style come into contact with the underside of the insect, as in many Fabaceae. In the nototribic form, the stamens and style contact the dorsal parts of the insect. Families with characteristically

nototribic flowers include the Lamiaceae (mint family) and Orchidaceae. This, of course, is of interest in the context of this project, as evidence is presented that the (nototribic) flowers *Diuris* orchids mimic the (stenotribic) flowers of peas of the tribe Mirbeliae.

The pea flowers of the Fabaceae are of the "flag blossom" type. These are of particular importance in this study as bee-pollinated "egg and bacon" pea flowers mentioned above are thought to be the abundant "model" species in the deceptive pollination system of *Diuris* orchids. Therefore, an understanding of the structure and function of these flowers is fundamental to pollination studies of these orchids. Pea flowers have a more or less tubular 5-lobed calyx and 5 petals. The uppermost petal is often enlarged and conspicuous and is known as the "standard". Below this is a pair of petals called the wings. Below and between the wing petals are another connate pair known as the keel. These are fused along their adjacent margins and are pressed and folded over each other to form a boat-like structure which encloses the 10 stamens, superior ovary and stigma. When a bee orients itself to a pea flower, it grasps the keel of the flower and probes for nectar at the base of the standard petal. Often a visible guide can be found at the base of the standard petal, but in some pea flowers these cues occur in the ultraviolet (Kay 1987). At the same time, pressure is applied to the keel, resulting in the fused or clustered, free stamens popping up and contacting the abdomen of the bee, thus permitting access to the pollen. Here, the bee is displaying behaviour adaptive to the exploitation of these flowers, resulting in mutual benefit to the flower i.e. pollination. Insects, such as small butterflies and syrphid flies (Diptera: Syrphidae - flower visitors known as 'hover flies') that lack adaptive anatomy and behaviour for pollination of these flowers may be seen to wander about the flower without accessing

the rewards (personal observation of Australian species in bushland). In this thesis, it is noted that ultraviolet nectar guides are frequently important in the tribes Mirbelieae and Bossiaeeae in Australian Fabaceae (Gross 1990; Gross 1992; Gross *et al.* 2000). These pea groups are often pollinated by bees specialising in pea flowers, such as the bee genus *Trichocolletes* (Hymenoptera: Colletidae). Importantly, as will be shown for *Diuris maculata* (see Chapter 2), analogous visual cues can be found in this orchid to those found in putative pea "model" flowers, a finding supportive of floral mimicry.

5. Special Features of Orchid Flowers

The orchid flower in structure at first seems quite unlike flowers of other plants, but shows a number of specialised modifications of a monocot flower such as a lily. Flower parts are in groups of three, with two perianth whorls. One member of the inner perianth whorl is usually highly modified into labellum, which often functions as a landing platform for pollinating insects.

Additionally, and perhaps most important, is the reduction and re-arrangement of the male and female sexual organs into a single structure known as the column. In most orchids the pollen is concentrated into pollinia (Darwin 1877; Johnson and Edwards 2000) which usually attach to a pollinating insect via a sticky pad called the viscidium. Pollinia have evolved independently in just two highly evolved plant families: the Orchidaceae and the Asclepiadaceae. The column and pollinia organisation favour the removal and transfer of outcrossed pollen in large pollen loads. This in turn permits fertilisation of large numbers of ovules and the formation of very large numbers of seeds.

The orchid seed is highly reduced and comprises little more than an embryo and seed coat (Pridgeon 1992). The tiny, light seeds float on air currents and are widely distributed. Lacking an endosperm, orchid seeds depend on infection by a mycorrhizal fungus to germinate.

The orchid family (Orchidaceae) is possibly the largest and is certainly the most diverse of all plant families. The basic architecture of the orchid flower has been become so endlessly adapted as to defy the imagination (Dressler 1990).

6. Deceptive Pollination is Common in the Orchid Family

Approximately one third of orchids have been estimated to employ deceptive pollination strategies (see Johnson 2000). An important feature of many orchids which employ deceptive pollination is mimicry, which can often be remarkably precise. Two main kinds are found: food source mimicry and sexual deception. This project is concerned with testing ideas of food source mimicry in *Diuris* (Orchidaceae), which show a visual resemblance to 'egg and bacon' peas of the tribes Mirbeliae and Bossiaeae.

7. Batesian and Batesian-type Mimicry in Plants, Especially Orchids

I have discussed many basic concepts of insect and floral features adaptive for pollination above. It is now appropriate to look at a specialised pollination mechanism where flowers offering no reward are pollinated by insect visitors to abundant, rewarding "model" flowers by a type of floral mimicry where the mimic resembles a reliable food source and by deception causes the insects to visit the non-rewarding

mimic. This mimicry system in flowers is known as a form of Batesian mimicry, but this is an adaptation of the original concept.

The term Batesian mimicry honours Henry Walter Bates, an amateur naturalist and entomologist of the 19th century. Bates spent 11 years in the Amazon region where he made many observations and collections of butterflies (Bates 1862). He noted that Heliconid butterflies, which were conspicuous and slow-flying were nevertheless avoided by predators. These butterflies are poisonous and their display is a warning to predators. He noted that unrelated Pierid butterflies which occurred together with the Heliconids often could not be distinguished from them in flight, and their similarity was often so close that an experienced entomologist was often fooled. Bates proposed that the Pierid butterflies, which were scarce, edible species, mimicked the common and toxic Heliconid butterflies, thus avoiding predation. A similar concept in entomology is Mullerian mimicry, where a guild of (frequently butterfly) species may be of similar size, coloration and offensiveness, and co-operatively reinforce a warning display.

Dafni (1992) defined Batesian mimicry in plant pollination as occurring where a rare species offers no reward and mimics flowers of a more abundant species that does offer a reward. On the face of it, Batesian mimicry in plants would seem to be very different to that described above in insects. However, although the effect of Batesian mimicry in plants is attraction of a pollinator rather than repulsion of a predator, the ecological conditions favouring the evolution of such mimicry are identical (Dafni. 1984; Ackerman 1986). Deceptive pollination is divided into categories of "nutritive deception" (food source mimicry) and "reproductive deception", which includes numerous instances of orchid pseudocopulation (e.g. Kullenberg 1961; Mant 2005) .

Dafni (1984) described Mullerian and Batesian mimicry in pollination of plants. Mullerian mimicry does not involve deception, but is instead a partnership of species of similar appearance, which increase the "effective density" of floral display and thus is mutually beneficial to participating species. An example of Mullerian mimicry in a group of related flowers are Australian pea flowers of tribes Mirbelieae and Bossiaeeae where a number of co-occurring species appear very similar (Gross 1990; Gross 1992). Similarity in appearance is probably a result of constraint in floral form of a suite of plesiomorphic characters despite a long period of evolutionary radiation (Wojciechowski 2003), Such a constraint is not incompatible with Mullerian mimicry, but may help explain its abundance with these pea flowers..

A basic feature of Batesian mimicry, as generally understood in both animal and plant systems, is that the mimicry is primarily visually based. Dafni (1992) and Johnson *et al.* (2003) defined these conditions for Batesian mimicry being that the mimic and model should co-exist, that the mimic should occur at a much lower frequency than the model, and that the mimic should resemble the model to the extent that the conditioned signal receiver (the pollinator(s)) is unable to distinguish them, and that the mimic should have higher fitness when it occurs with the model. Conventionally, the mimic is expected to lack a floral reward, Presence of a reward has been shown to increase pollen transfer efficiency (Peter and Johnson 2009) and increased pollination success. These conditions would place the mimic in a position of dependence on the association with the model, but this last aspect is often the most difficult to demonstrate if the mimic is never found growing in isolation from the model. In orchids Batesian mimicry is associated with low reproductive success (e.g. Johnson 2000; Beardsell *et al.* 1986), but is considered to favour increased outcrossing (see Johnson 2000).

Batesian mimicry in plants is exemplified within the Orchidaceae, in which as many as one third of all species are estimated to not produce floral rewards of any kind (van der Pijl and Dodson 1966). Examples of mimicry tend to be anecdotal (e.g. Cingel 2001 for *Diuris*). Studies in which the above conditions for Batesian mimicry are formally studied are relatively few. Nevertheless, some remarkable suggested examples have appeared in the scientific literature. Kjellsson *et al.* (1985) proposed floral mimicry of two orchids, *Dendrobium infundibulum* and *Cymbidium insigne* of the white flowered *Rhododendron lyi* in Thailand. This rhododendron has large white flowers which are somewhat zygomorphic in form, and with a yellow patch on one of the petals. The two comparatively rare, sympatric orchid species both have large white flowers with a yellow patch on the lip (labellum). The bumblebee *Bombus eximius* was shown to pollinate the rhododendron. Orchid pollinaria of the two orchid species were found attached to bumblebees at the site, but deposition of orchid pollinaria on a bumblebee was only seen once, in *D. infundibulum*.

Other cases where a specific pollinator is proposed to pollinate orchids by food source deception include examples from South Africa. Johnson *et al.* (2000) proposed that the non-rewarding orchid *Disa pulchra* is a Batesian mimic of *Watsonia lepida* (Iridaceae), which is pollinated by a Tabanid fly *Philoliche aethiopica*. Similarly, another *Disa* species, *D. ferruginea* (Johnson 1994) was proposed to be a Batesian mimic of the butterfly-pollinated *Tritoniopsis triticea*, also a member of the Iridaceae. Johnson proposes that both these pollinator types forage mainly by visual cues (Johnson *et al.* 2003).

The above examples, involving specific pollinators and very close visual resemblance of the mimic flower to the model would seem quite convincing. Other, looser examples of mimicry have also been proposed. For example, Dafni and Ivri (1981) proposed floral mimicry between the orchid *Orchis israelitica* and the widespread model, the bulbous plant *Bellevallia flexuosa*. These two plant species show close visual similarity. *B. flexuosa* attracts a range of pollinators at different sites, and the pollination of the orchid similarly was shown to be flexible as regards pollinators, depending on where it occurred.

Sugiura *et al.* (2002) studied the pollination of the ladyslipper orchid, *Cypripedium macranthos* var. *rebunense* on Rebun Island, Hokkaido, Japan by queen bumblebess, *Bombus pseudobaicalensis*. This orchid satisfied many of the expected conditions for Batesian mimicry described above. The queen bees pollinate the nectar-producing flowers of *Pedicularis schistostegia* (Orobanchaceae). It is interesting that the orchid resembled the model in colour, but not floral form. The early flowering of the *Pedicularis*, with few other flowers at the time of the appearance of the queen may contribute to the evolution of floral mimicry in this scentless orchid.

Beardsell *et al.* (1986) studied the pollination of the nectarless terrestrial orchid *Diuris maculata* at Fern Tree Gully, near Melbourne, Victoria, Australia. I have already discussed this above. This orchid was shown to co-occur with native legumes in the genera *Daviesia*, *Pultenaea* and *Dillwynia*, colloquially known as "egg and bacon" peas, which were proposed to be models of the pea-like orchid flowers. Evidence was presented for the orchid to be pollinated by the bees of the genera *Trichocolletes* and *Leioproctus*, plus a wasp *Gasteruption* sp., which were visitors of pea flowers. Clearly,

this form of pollination was of a Batesian-type, but somewhat more generic in the sense of multiple models and pollinators being involved, particularly when one considers the widespread distribution of this species.

Bernhardt and Burns-Balogh (1986) studied the pollination of *Thelymitra nuda*, a sun orchid. Orchids in the genus *Thelymitra* are common Australian terrestrial species with relatively large flowers that only open in sunlight and are also notable for the unspecialised labellum, with the result being that the flowers have an actinomorphic appearance. Female bees of the genus *Lasioglossum* (Halictidae) were seen to curl their bodies around the four-lobed tip of the staminodal complex of the orchid flowers. This was proposed to be due to mimicry by the orchid of poricidal anthers of flowers offering only a pollen reward in the 'Liliaceae' genera *Dichopogon* and *Thysanotus* (now placed in Laxmanniaceae).

These examples of floral mimicry studies, which by no means comprise a comprehensive coverage of the subject, vary in the specificity of modelling, and focus on the pollination mechanism of individual orchid species. Steven Johnson, who has been one of the most active researchers in the field of floral mimicry has noted the lack of comparative perspective in pollination studies and has attempted to address this with a study mapping pollination mechanisms onto a phylogeny for the African terrestrial orchid genus *Disa* (Johnson *et al.* 1998). The pollination mechanisms of this orchid genus have been extensively studied. Johnson *et al.* (1998) undertook a phylogenetic study of the genus based on morphological characters and then mapped pollination mechanisms to the cladogram generated. *Disa* is remarkable for the diversity of pollination mechanisms, which include various bees, butterflies, wasps, and flies of

different families. There are likely very few plant genera with comparable pollination diversity. In response to a few dominant pollinators, species within clades in *Disa* have shown remarkable adaptation to a diversity of pollinators. The cladogram clearly shows that adaptation to various pollinators has occurred repeatedly in *Disa*. The adaptive nature of floral traits can result in related plants within a genus such as *Disa* showing a diversity in floral form, whilst unrelated plants may show convergence in floral form in forming a pollination guild (Johnson *et al.* 2003).

8. Advances Beyond Descriptive Studies

The natural tendency in studies of floral mimicry would seem to be a process of anecdotal observation of possible mimicry, followed by basically descriptive research studies with attempts to satisfy the criteria set out by workers such as Dafni (1992), who has been one of the leaders in this field. The outcome of such research tends to be a more or less convincing proposal of floral mimicry to add to a slowly expanding catalogue of such studies.

Some theoretical questions remain problematic. One of the basic features of Batesian-type floral mimicry is a low reproductive success. Studies consistently report pollination success of fewer than 20% of flowers, in contrast to much higher reproductive success of rewarding species. Proposed benefits of mimicry tend to focus on some key benefits including:

1. Increased outcrossing. Experimental evidence presented by Johnson *et al.* (2000) in a study of the orchid *Disa pulchra,* a non-rewarding putative mimic of the rewarding iris *Watsonia lepida,* showed that flies did not discriminate between

the flowers of the two species. However, pollinators spent more time on inflorescences of the rewarding species, an observation suggested to promote outcrossing of the mimic. Studies such as Beardsell *et al.* (1986) for *Diuris maculata* and Johnson *et al.* (2003) for *Brownleea galpinii* ssp. *major* demonstrate a higher experimental seed set in flowers that were outcrossed compared to those self-pollinated in bagging experiments.

2. The idea that lack of floral reward has significant energetic cost savings compared with provision of floral reward is frequently proposed. This point is contentious and considered by many to be doubtful.

Johnson *et al.* (1999) experimentally manipulated flowers of non-rewarding orchids *Orchis mascula* and *O. morio* by artificially adding a nectar reward. A nectar reward was shown to result in queen bumblebees remaining longer on the nectar-containing flowers than on non-rewarding flowers of the same species. These *Orchis* species possess pollinaria which need to undergo bending and therefore have a time delay before pollination is possible by transfer to another flower. Nevertheless Johnson *et al.* (1999) propose that the offering of a reward would result in increased geitonogamy. As fruit set in orchids is usually pollinator-limited the authors suggest additional explanations may have to be sought to explain deception.

A general observation (and not too surprising) is that deceptive orchids consistency show fruit set significantly above zero. It is well known that orchid capsules generally produce prodigious amounts of tiny seeds. No one seems to have asked the question of whether reproductive success in the orchids is seed (and recruitment)

limited i.e. whether sufficient seeds are produced to find all suitable habitat. This question would be difficult to answer.

Another issue worthy of consideration is relative costs of display versus reward. Johnson *et al.* (1999) suggest that resources may be more favourably invested in floral display than reward. Of course, resources invested by the orchids in floral display, including nectar (Luyt and Johnson 2002), can to a degree be reabsorbed after flowers finish and potentially allocated into seed production. In contrast, nectar, if it is taken away can no longer be used by the plant. Seed production is undoubtedly of high cost to plants (although orchids do not invest much nutrient in each seed). Mimicry by food source deception could be viewed as a means of maintaining a suitably small resource allocation into seeds.

Another important consideration is that Batesian mimicry in orchids may allow them to exist in habitats dominated by plants with particular abundant pollinators. There may simply be no other pollinators around in significant numbers to effect pollination. The evolutionary question for many orchid species that may adapt to these habitats may therefore be reduced to comparative benefits of Mullerian versus Batesian mimicry.

9. Phylogenetic Analysis and Pollination Ecology

Traditionally classification schemes for organisms were based on comparisons of features, but using intuitive methods to a great degree. Perceived overall similarity according to such methods would suggest the degree to which organisms might be related.

The German entomologist Willi Hennig was interested in developing a method for implementing Darwin's ideas into a classification scheme based on evolutionary genealogy (Hennig 1966; Kitching 1998). He developed his "phylogenetic systematics" as a means of reconstructing these relationships using empirical means. These methods became known as cladistic analysis. The aim of this method is to reconstruct phylogenetic relationships in the form of a hierarchical, branching tree diagram, or cladogram. The basic method is to formulate similarities into discrete characters that can be scored as "absent" or "present". Cladistic analysis aims to find the cladogram that accounts for the distribution of the scored characters in the simplest way, by minimizing the number of hypothesized transformations between the states in each character. This criterion is known as parsimony, the underlying idea being that the simplest explanation of the data is the most robust solution to a phylogenetic problem. The phylogenetic trees produced by cladistic methods are hypotheses of evolutionary relationships. More sophisticated statistical approaches such as maximum likelihood and Bayesian statistics have been used to develop specialised techniques for analysing particular classes of data (Page & Holmes 1998), especially for DNA sequence alignments, but parsimony remains the criterion for phylogenetic analysis that is applicable to the widest range of comparative data. Modern systematics is increasingly informed by cladistic analysis in an attempt to construct classification schemes that reflect phylogeny.

A detailed account of cladistic methods will not be given here. However, the idea of mapping biological features of groups of organisms onto a phylogenetic tree is an appealing one. Insights gained from such an approach might include a hypothesis as to the ancestral pollination mode for a group of organisms and which features may be

more recently derived. Cladistic analysis may also reveal that a biological feature may have arisen more than once independently (homoplasy). In the case of *Diuris*, phylogenetic analysis (presented in Chapter 5) suggests that Batesian-type mimicry may be an ancestral feature, apparently retained with modification by many members of the genus, but with some exceptions.

Diuris maculata is the only species in the genus for which a detailed pollination study had been undertaken prior to this project. The circumscription of this and a number of other species is somewhat controversial. A cluster of species including *D. maculata*, *D. goonooensis*, *D. platichila, D. semilunulata* and *D. pardina* clearly are closely related, and share similar floral morphology and habitat preferences (see Bishop 2000). Similar issues apply to the *D. punctata* complex of species. Bishop could fairly be described as a "lumper": inclined to group closely related species in synonomy, whilst others tend to split taxa into distinct species. The great variability within and between populations of *Diuris* makes traditional morphology-based systematics especially difficult and more "objective" molecular approaches therefore hold promise in clarifying the situation. Molecular systematics can be expected to contribute to pollination studies in a number of important ways:

1. By revealing the structure of "clades" within *Diuris*: groups of related species which share a common ancestor. Genetically similar species can be resolved from those which may appear similar through homoplasy i.e. which may have converged towards similar floral and/or vegetative form through similar selective pressures resulting in shared pollinators. Focusing pollination studies of key species within clades would be the ideal means of resolving pollination patterns within a large group.

2. Genetic variability within clades can be expected to vary for a number of reasons. Recent and rapid species radiation can be expected to result in poor resolution of species due to insufficient time for DNA variation to arise. Conversely, high resolution of species within clades would result from species having evolved steadily over a long period, or perhaps rapid evolution followed by stasis.
3. Information from cladistic analysis of the orchid genus *Diuris* combined with evidence of historical climate change, evolution of Australian Mirbeliae and Bossiaeae legume groups and pollinators can be expected to contribute insights into how present day pollination patterns may have arisen.

10. Pollination of *Diuris*

The genus *Diuris* (Orchidaceae) comprises at least 61 species, according to the compilation of Clements **(http://anbg.gov.au/cpbr/cd-keys/orchidkey/html/currentspecies.html).** They are almost entirely restricted to Australia, with the exception being *D. fryana* which occurs in Timor. Species are well represented in both the south east and south west of the country Bishop 2000; Jones 2006). They are often found in open forest and woodland with a grassy understorey, often dominated by kangaroo grass, *Themeda australis* (Poaceae). The plants are seasonally deciduous, surviving as dormant tubers for part of the year (usually the summer). They have linear, grass-like leaves and are only conspicuous when in flower. The flowering period is short, about 4-5 weeks, and it is very difficult to locate them in their habitat outside of the flowering period.

The majority of *Diuris* species have flowers which show resemblance to pea flowers encountered in the Australian bush, particularly 'egg and bacon' peas such as those in the genera *Pultenaea*, *Davesia* and *Bossiaea*. Flowers are arranged on an upright raceme, usually of 5-8 flowers. Many species have a yellow base colour (e.g. *Diuris maculata* – see Chapter 3, Figure 3.1) and may appear remarkably like pea flowers. Others, with a white to pink base colour, often have similar floral form to the yellow species (e.g. *Diuris alba* – see Chapter 4. Figure 4.1). Others, such as *Diuris chryseopsis* (not illustrated) have less obvious pea resemblance. *Diuris maculata* flowers will be described as a representative example of this genus. The dorsal sepal is uppermost and is rather prominent and rounded and can be compared with the standard petal of a pea flower. The lateral sepals form the 'two tails' upon which the genus name *Diuris* is based. These are relatively short in *D. maculata* and being darkened in colour are fairly inconspicuous. The labellum of *D. maculata* is three lobed, and in combination with the dorsal sepal gives the effect of a pea-like flower. The lateral sepals are brightly coloured and prominently displayed. They do not contribute to the 'pea-like' appearance, but may help to give the effect of a massed floral display. The tip the column is clearly visible in *D. maculata*. In particular, the disc-shaped sticky viscidium of the pollinarium is exposed so that it can become attached to the head of a bee visitor. In this species it is easy to see if the pollinarium has been removed (see Chapter 3, Figure 3.2b and c). Pollinaria have the appearance of a small pair of dumbbells (see Chapter 3, Figure 3.2a).

Van der Cingel (2001) reviewed the pollination of *Diuris*. Most published observations on pollination offer very fragmentary evidence and are far from conclusive. By far the most comprehensive study to date is by Beardsell *et al.* (1986) on

the pollination of *Diuris maculata* at Fern Tree Gully, near Melbourne. They concluded that *D. maculata* is most likely a floral mimic of sympatric legume shrubs: two *Daviesia* spp. and *Pultenaea scabra* and they provided data showing that the pollination system of the species at that site meets most of the established criteria for Batesian mimicry (Dafni 1984). They showed that the orchid was found in spatial and temporal association with the legume shrubs (the putative models), which were much more abundant than the putative mimic - the orchid. Although observations of insect visits to the orchid were rare, hymenopteran insects (native bees and a wasp species), the main floral visitors of the legumes, were frequently observed to be carrying orchid pollinaria on their faces. The orchid was shown to possess visual similarity to the legume flowers, and was stated to lack nectar (although this was not tested). Their interpretation was that the visitors to the orchid, which were shown by pollen analysis to visit mainly legumes, were deceived by food source mimicry of the legume flowers. Pollinaria were shown to be removed from orchid flowers at a higher frequency than were transferred to subsequent flowers, resulting in a quite low level of fruit set from opened flowers of 19%. One of the accepted criteria for Batesian mimicry not met in this study was a demonstration that reproductive success would be greater in the presence of a putative model than in its absence, but few studies of floral mimicry have been able to meet this criterion (Roy and Widmer 1999). The study by Beardsell *et al.* (1986) was thus an important advance on the previously largely anecdotal observations of pollination within *Diuris*.

The term Batesian mimicry originally referred to the observations of Henry Bates during his 11 years in the Amazon (Bates 1862). He observed that some butterfly species appeared to mimic toxic species of butterfly and by doing so avoided predation.

Many examples have since been found of Batesian mimicry in insects and other animals. The concept has been extended to include deceptive pollination in plants where a relatively rare species that does not provide a food source as a reward mimics an abundant species that does offer a food reward, and consequently is visited by the pollinator(s) of the rewarding species. This form of Batesian mimicry has been suggested to play a part in the pollination of a number of orchid species (Dafni and Ivri 1981; Kjellsson *et al.* 1985; Johnson 1994; Johnson *et al.* 2000; Sugiura *et al.* 2002). Such orchids tend to be quite rare and have restricted distribution. The above examples mostly feature remarkable visual similarity of the orchid(s) to a single species of putative model that is common and widespread . In contrast, Beardsell *et al.* (1986), in their study of *Diuris maculata* pollination at one site, suggested several putative model species, and argued that the orchid therefore possesses more generalised similarity to these legume flowers, but does not seem to be a specific mimic of any particular species. Therefore, *D. maculata* has been suggested to be a "guild mimic". Such a pollination system shows elements of Batesian mimicry (Dafni and Bernhardt 1990), but the model is of a general type rather than a specific species. Such a pollination system would have benefits for a species such as *D. maculata,* which is common and widespread (Bishop 2000), in contrast to the above examples of putative Batesian mimics.

Since the majority of *Diuris* species have strongly pea-like flowers it is reasonable to infer that similarities in pollination may occur in most, if not all, species in the genus. However, prior to this project, a comprehensive pollination study of *Diuris* species had been undertaken on just one species, in one locality (Beardsell *et al.* 1986), which could not be considered sufficient to understand the pollination of even this one

species, let alone provide much insight into pollination of the genus as a whole. At the very least pollination studies of representatives of the main taxonomic groups within the genus need to be undertaken in order to resolve patterns in its pollination biology. No infrageneric classification of *Diuris*, based on an explicit phylogenetic analysis, existed prior to this study, so a systematic study of *Diuris* was also considered helpful to more focused studies of pollination within lineages of the genus.

Given the highly specialised pollination systems of orchids, pollination studies are central to decisions about the conservation management of native orchids. Studies of species populations in a relatively undisturbed state can answer basic questions of pollination processes. A detailed understanding of a species' pollination biology can be used in conservation management to maintain crucial components, such as viable pollinator populations, and to detect impaired pollination.

This thesis is primarily concerned with increasing knowledge of *Diuris* pollination. A logical starting point was to extend the work of Beardsell et al. (1986) in *D. maculata* pollination. Their study was the most detailed to date on *Diuris* pollination when I started this project, but was focused on pollination of this one species at just one site. *Diuris maculata* is a widespread and common species (Bishop 2000). It was reasonable to suggest that a similar pollination mode may occur within the many populations of this species, but it is necessary to test such an assumption. Even if the orchid occupies similar ecological niches throughout its range, the specific putative model(s) and pollinator(s) must vary considerably. Thus the pollination of this orchid was expected to be considerably more flexible than comparable studies of orchid Batesian mimicry. As *Diuris maculata* populations occur within the Sydney region, this

provided an opportunity to test the findings of Beardsell et al. (1986) to discover whether similar floral mimicry is found despite expected differences in pollinators and putative model species.

In addition to the application of the methods of Beardsell *et al.* (1986), advances in technology have opened the way for additional forms of evidence. Many flowers, including Australian legumes from the subtribes Mirbeliae and Bossiaeeae, possess an ultraviolet colour component to their flowers that is visible to insects, particularly hymenopteran visitors (Kay 1987; Gross 1990). Native pea flowers, such as those of *Pultenaea*, *Daviesia* and *Dillwynia* have zygomorphic flowers specially adapted to hymenopteran visitors. While the flower has an overall colour that includes an ultraviolet component, these flowers also possess an ultraviolet-absorbing patch at the base of the standard petal, which provides a colour contrast visible to insects, and which functions as a nectar guide. As it has been suggested that the flowers of *Diuris maculata*, and presumably also many other *Diuris* species, are mimics of pea flowers of this type, then it is to be expected that the *Diuris* orchid flowers should possess similar patterns, visible to insects in the ultraviolet range. Such patterns can be detected photographically using ultraviolet sensitive film and a filter to exclude visible light (Williams and Williams 1993; Indsto and Weston 1999). This technique was known at the time of the Beardsell *et al.* (1986) study, but as this was prior to the publication of the paper by Kay (1987), the ultraviolet patterns of native pea flowers would not have been widely known.

Native pea flowers produce nectar, but often in very small quantities (Gross 1990), that require some skill to detect. Little data currently exists to support the widely

held belief that *Diuris* flowers lack nectar, so the application of the methods of Gross (1990) can be of help in clarifying this question, which has importance in testing for mimicry in these orchids.

DNA methodologies have been employed in this project for two main reasons. DNA fingerprinting of orchid pollinaria remnants removed from the heads of putative pollinating insects was used to identify *Diuris* species where more than one species of *Diuris* may be sympatric, or where pollen might potentially have come from a population some distance away. Also, a phylogenetic analysis of the genus was undertaken with the aim of mapping pollination to species groups and to allow insight into the distribution amongst species of synapomorphies pertinent to pollination and how these may have affected the success of these species.

11. Summary of Main Aims / Hypotheses Tested in this Project:

* The Beardsell *et al.* (1986) study proposes food source mimicry of pea flowers for *Diuris maculata* at one site in Melbourne. The pollination systems of populations of this and other *Diuris* species was examined.

* *Diuris* flowers have been suggested to lack nectar. Tests were conducted on *Diuris maculata* and other *Diuris* species to document nectar production status.

* Expanded studies of pollination systems in *Diuris* to explore the extent and nature of mimicry.

* Development of DNA-based methods for identification of *Diuris* species using AFLP and ITS DNA sequences (Internal Transcribed Spacers of Ribosomal DNA).

* A cladistic analysis of *Diuris* based on AFLP and ITS conducted to reveal species relationships, and also to provide insights into the evolution of pollination mechanisms.

Chapter 2

Highly Sensitive DNA Fingerprinting of Orchid Pollinaria Remnants Using AFLP (Amplified Fragment Length Polymorphism)

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Prologue

This chapter appeared as a published paper (Indsto, JO, Weston, PH, Clements, MA and Whelan, RJ (2005) **Highly sensitive DNA fingerprinting of orchid pollinaria remnants using AFLP**, *Australian Systematic Botany* **18**, 207, 207-213). The chapter presented here is essentially as published with minor changes. Two corrections to the original paper are corrected in this version (see *Australian Systematic Botany* (2005) **18**(5) 473-473). References to papers in preparation at the time of publication of this paper now appear as citations. The first author (JI) was responsible for the original idea and laboratory experiments. Peter Weston provided early assistance in getting AFLP working and provided extensive guidance in the cladistic analysis.

Abstract

Numerous Australian terrestrial orchid species in the genus *Diuris* may be pollinated by food source mimicry. In our field studies, direct observations of orchid-pollinator interactions were rare, but native bees were frequently captured carrying orchid pollinaria, or pollinaria remnants. Sometimes, pollinaria remnants were minimal and included only the viscidium, a sticky pad that was often highly persistent. Confirmation of such tissue as being of orchid source, and of a particular species can aid pollination studies. DNA-based methods that may identify more or less intact orchid pollinia are available, but extremely small and degraded samples can pose technical challenges. We have developed an AFLP (Amplified Fragment Length Polymorphism) protocol for such difficult samples that offers some significant advantages over direct PCR-based analysis. We simulated AFLP profiling of very low-DNA samples using DNA template from serial dilutions. A DNA sample range from 6.4 picograms to at least as high as 100 nanograms (15,500 fold range) all yielded AFLP fingerprints. The practical application of this inherent sensitivity of AFLP is demonstrated by the identification of remnants of orchid pollinaria sampled from bees, presented here as a case study. It is expected that this approach will find many applications where sample DNA is limiting, or possibly where pollen of similar appearance may comprise species mixtures.

Introduction

Beardsell *et al.* (1986) presented evidence suggesting that *Diuris maculata,* an Australian species of terrestrial orchid, is a floral mimic of legume shrubs in the genera *Daviesia* and *Pultenaea.* We suspect that pollination may be similar in many *Diuris* species and seek to test this hypothesis more broadly. Directly observing pollination of

an orchid suspected of being a floral mimic requires considerable patience as visits by putative pollinators are often infrequent (Beardsell *et al.* 1986). However, in field observations of various *Diuris* populations and species, we frequently encountered putative pollinating bees with obvious orchid pollinaria attached to their faces, indirect evidence that the bees had visited orchids (Fig 2.1*b*).

It is quite common for different *Diuris* species to occur and flower together. This may lead to uncertainty as to the species source of orchid pollinaria attached to putative pollinating insects. Identification of such orchid pollinaria is potentially one of the most valuable means of elucidating pollinator behaviour and is often only possible using DNA-based methods. We also commonly observed with insects putatively carrying *Diuris maculata* pollinaria remnants that little or no pollen remained and only desiccated remnants of the viscidium, the sticky pad that holds the pollinaria in place, remained on the bee, thus providing a challenge for analysis (see Fig 2.1*d*). In this study we show that a modified AFLP protocol can successfully be used to identify such samples and discuss the efficacy of this approach in relation to alternative molecular techniques.

We have used molecular data from AFLP and ITS sequencing to cladistically analyse species relationships of orchids in the genus *Diuris* as part of an investigation of their pollination biology strategy for pollination biology (Indsto *et al.* 2006; Indsto *et al.* 2007). We have found AFLP to be a suitable independent source of characters in this cladistic study. The flowers of *Diuris* species often show a marked similarity to legume flowers of such genera as *Pultenaea*, *Bossiaea*, *Daviesia* and *Dillwynia* (colloquially known as egg and bacon peas). The similarity of these legumes reflects a pollination

guild: long-term selective retention of homologous bee visual cues to the extent that sympatric egg and bacon legumes can be considered to mimic each other. In turn, similarity of the putative orchid mimics most likely reflects floral convergence to legume guilds, and can be best termed guild mimics (Dafni and Bernhart 1990).

A cladistic anlaysis can inform a pollination study of a large orchid group in a number of ways. For example, a group of closely related species, with shared floral features, may reflect recent evolutionary radiation with an underlying innovation in floral biology, or conversely, species of highly similar appearance, but which may be genetically more dissimilar, may reflect conservation of floral form over a long period. In other words cladistic analysis may be used to reconstruct the character phylogenies of features that are functionally important in pollination. Moreover, molecular dating techniques may be used to estimate the relative or even absolute timing of evolutionary changes. Perhaps most importantly, cladistic analysis provides a framework for identifying the diagnostic molecular characters of species and species groups.

The details of our cladistic analysis can only be outlined here. We have developed an AFLP profile library of the main taxa within *Diuris* that are found within about 200 km of Sydney, plus several samples kindly supplied by volunteers from further afield. We found three very distinct clades of *Diuris* species: a clade comprising *Diuris sulphurea* only, another comprising species closely related to *D. maculata*, and another of species closely related to *D. punctata*. AFLP and ITS sequencing has been found to resolve only a few of the species within these clades. We expected AFLP to show higher resolution than ITS, as several studies have shown very high resolution using AFLP, even to the point of parentage testing in a population of plants (Krauss 2000). Fortuitously, in field studies where more than one species of *Diuris* have been

found flowering together, the species present were always ones that could be distinguished using AFLP.

Widmer *et al.* (2000) used a CTAB-based procedure to extract PCR-quality DNA for ITS1 sequencing from orchid pollinaria removed from insects, many of which had been kept for some years in museum collections. Undoubtedly, the strategy they employed was as efficacious for their samples as alternatives, including AFLP. For several of our more challenging samples we expected DNA extracts with very low yields and probably less than 1 ng in total, and even this DNA likely to be somewhat degraded. We present evidence that AFLP not only has the capability of analysing such samples, but has certain advantages over alternative methods.

AFLP, as described by Vos et al. (1995), typically employs 500 ng in the first, restriction digest step, so at first consideration would appear a most unsuitable approach where DNA is in limited supply. However, Coyle *et al.* (2003) reduced DNA for restriction digestion to 20 ng without significant protocol modifications. As we show below, DNA template for this first critical step can be reduced substantially further.

The AFLP procedure (Vos *et al.* 1995) has achieved considerable popularity with many studies in fields such as systematics (Le Thierry d'Ennequin *et al.* 2000), marijuana cultivar identification (Coyle *et al.*2003) and determination of parentage in pollination ecology (Krauss 2000). In systematic studies, AFLP, even if not employed as the primary investigative tool, has merit in supporting phylogenetic results based on sequencing of nuclear and/or chloroplast genes (Hedren *et al.* 2001). Vos et al. (1995) showed the potential forensic application of AFLP with dilutions of restricted, ligated

DNA in the range of 25 ng to 2.5 pg in pre-selective PCR amplifications resulting in an analysable final AFLP profile. In this study, we use experimental simulation to show the sensitivity of a modified AFLP protocol in obtaining DNA fingerprints from restriction digests and ligations of serial dilutions of DNA ranging from 6.4 pg to 100 ng: a range of ~15,500 fold. We extend the findings of Vos et al. (1995) by more completely simulating the start and completion of AFLP profiling with a dilute DNA sample. We then detail a protocol for the practical forensic application, with test samples, of AFLP through all steps of DNA extraction, restriction digestion and ligation, pre-selective PCR and finally selective PCR using a fluorescent primer. The AFLP approach involves several steps, each of which could be repeated if necessary, without all material being used up in any one step. This property, combined with sensitivity, makes it highly attractive for important and meagre samples.

Materials and Methods

We modified the AFLP procedure as described by Vos et al. (1995) as follows: (1) DNA extraction using the Qiagen Plant DNeasy Mini kit; (2) restriction digestion and ligation of highly diluted DNA samples (cf. 500 ng normally used); (3) use of undiluted restriction digest/ligation product directly for preselective PCR (cf. 20-fold dilution) with (4) much lower than usual preselective PCR template; (5) use of 2% formamide in PCR steps (Ranamukhaarachchi *et al.* 2000); (6) increase of cycle number in pre-selective PCR and (7) use of a touchdown PCR protocol in both pre-selective and selective PCR. Vos et al. (1995) previously used AFLP modifications (4) and (6) in their demonstration of AFLP using highly diluted pre-selective PCR starting template.

AFLP Simulation Experiment Using Varying Template Concentrations

We used a DNA dilution series to simulate the use of AFLP with samples of very low DNA yield, to explore the technical limits of AFLP. DNA extracts of two genetically orchid species, *Diuris alba* (from Yeppoon, Qld, Australia), a species belonging to a clade related to *Diuris punctata*, and *Diuris maculata* (from Scheyville National Park, NSW, Australia), a member of a distinct, different clade. Fresh whole flowers were desiccated in a Zip-Lock bag with silica gel for ~10 days at room temperature and then stored at -20° C until required. Individual dried flowers, each weighing about 10 mg, were added to 2 mL microfuge tubes with a few grains of acid-washed sand. The tubes were placed in 15 mL cryovials containing liquid nitrogen to ~20 mm depth and the frozen tissue ground using an autoclaved bamboo skewer. The Qiagen Plant DNeasy Mini Kit (Qiagen GmbH, Germany) protocol was followed without modification and DNA eluted into 200 µL AE buffer. The DNA yield was determined by spectrophotometry.

AFLP reagents, including restriction enzymes *Eco*R1 and *Mse*1 (New England Biolabs Inc., Beverly, MA, USA) and AFLP adapters and primers (Sigma-Genosys, Australia) were used as described by Vos et al. (1995), except that the *Eco*R1 selective primers were 5'-HEX labelled. A combined restriction digest and ligation was carried out. DNA was diluted to 1 µg in 10 µl $TE_{0.1}$ ($TE_{0.1}$ = 10 mM Tris pH 8.0; 0.1 mM EDTA pH 8.0) and a 5-fold serial dilution series prepared containing 500, 100, 20 and 4 ng and 800, 160 and 32 pg respectively in 10 µL $TE_{0.1}$. Reaction master mix (10 µL) was added, containing, for a 20 µL final volume, 0.5 µM *Eco*R1 adapter, 5 µM *Mse*1

adapter, 1 X T4 Ligase Buffer (New England Biolabs), 0.5 µg BSA, 50 mM NaCl, 2 U *Mse*1, 5 U *Eco*R1 and 1 U T4 DNA Ligase (New England Biolabs Inc.). The mixture was incubated at 37 C for 4 h. Restriction/ligation mix (4 µL) was used as pre-selective PCR template without prior dilution. This corresponds to pre-selective PCR DNA template of 100, 50 (previous diluted 1:1 in $TE_{0.1}$), 20, 4 ng and 800, 160, 32 and 6.4 pg respectively.

Pre-selective PCR was conducted in 20 µL volumes containing 200 µM dNTPs, 20 ng each of *Eco*R1 and *Mse*1 pre-selective primers, 0.5 µg BSA (Giambernardi *et al.* 1998), 50mM KCl, 10 mM Tris pH 8.5, 2.5 mM $MgCl_2$, 2% formamide (Ranamukhaarachchi *et al.* 2000) and 1 U *Taq* DNA polymerase. A Corbett Research FTS-960 Thermal Sequencer was used with 200 µL capacity tubes. A touchdown PCR protocol was employed with one cycle of 95° C for 3 min, followed by successive cycles of 95° C denaturation for 20 s, annealing for 30 s and 72° C extension for 2 min with the first annealing at 66° C and progressively reduced each cycle by 1° C until the touchdown annealing temperature of 56° C was reached. This was followed by a further 24 cycles with 56°C annealing, and a final extension of 72° C for 10 min. Half the pre-selective PCR product (10 µL) was run on a 2% agarose gel to check for a visible smear, indicative of successful amplification of many products of variable size (Fig 2.2).

The remaining 10 µL was diluted 20-fold with $TE_{0.1}$ and 4 µL used as template for selective PCR in 20 µL reactions containing 200 µM dNTPs, 60 ng each of the 2 base-pair selective primer combination 5'-HEX *Eco*R1-AC with *Mse*1-CT, 50 mM KCl, 10 mM Tris pH 8.5, 2.5 mM $MgCl_2$, 0.5 µg BSA, 2% formamide and 1 U *Taq* DNA

polymerase, and using the same protocol as for pre-selective PCR. An equal volume of denaturing loading dye of formamide containing 10 mM EDTA pH 8.0 and bromophenol blue was added and the samples heat denatured for 3 min at 95° C and snap chilled on ice. 2-3 μL was loaded on a 5% 29:1 acrylamide:bis gel containing 7.5 M urea and 0.6 X TBE and run in 0.6 X TBE at 40° C and 900V in a Corbett Gel-Scan 2000 DNA Analyser using He-Ne laser detection.

DNA Extraction and AFLP of Orchid Pollinaria and Pollinaria Remnants

Pollinaria from fresh flowers were removed using a small straw (see Figure 2.1*b*), placed in a 1.5 mL microcentrifuge tube, and stored at -20° C. DNA extraction of orchid pollinaria removed from flowers was similar to that used for whole flowers described above, except that an autoclaved plastic pellet pestle was used (to minimise losses) and DNA was precipitated in ethanol at -20°C overnight, after which it was spun at approximately 14,000 *g* for 15 min, and the DNA pellet washed with 75% ethanol. Finally the air-dried pellet was resuspended in 30 μL TE $_{0.1}$. DNA was similarly extracted for small remnants of orchid pollinaria were removed from the heads of four bees, but air-dried pellets resuspended directly in 20 μL restriction digest/ligation mixture and incubated as above for AFLP, including a water control.

Samples were subjected to three independent AFLP amplifications using three selective primer combinations: 5'-HEX-*Eco*R1-AC with *Mse*1-CT, 5'- HEX-*Eco*R1-AA with *Mse*1-CT and 5'-HEX-*Eco*R1-AA with *Mse*1-CG.

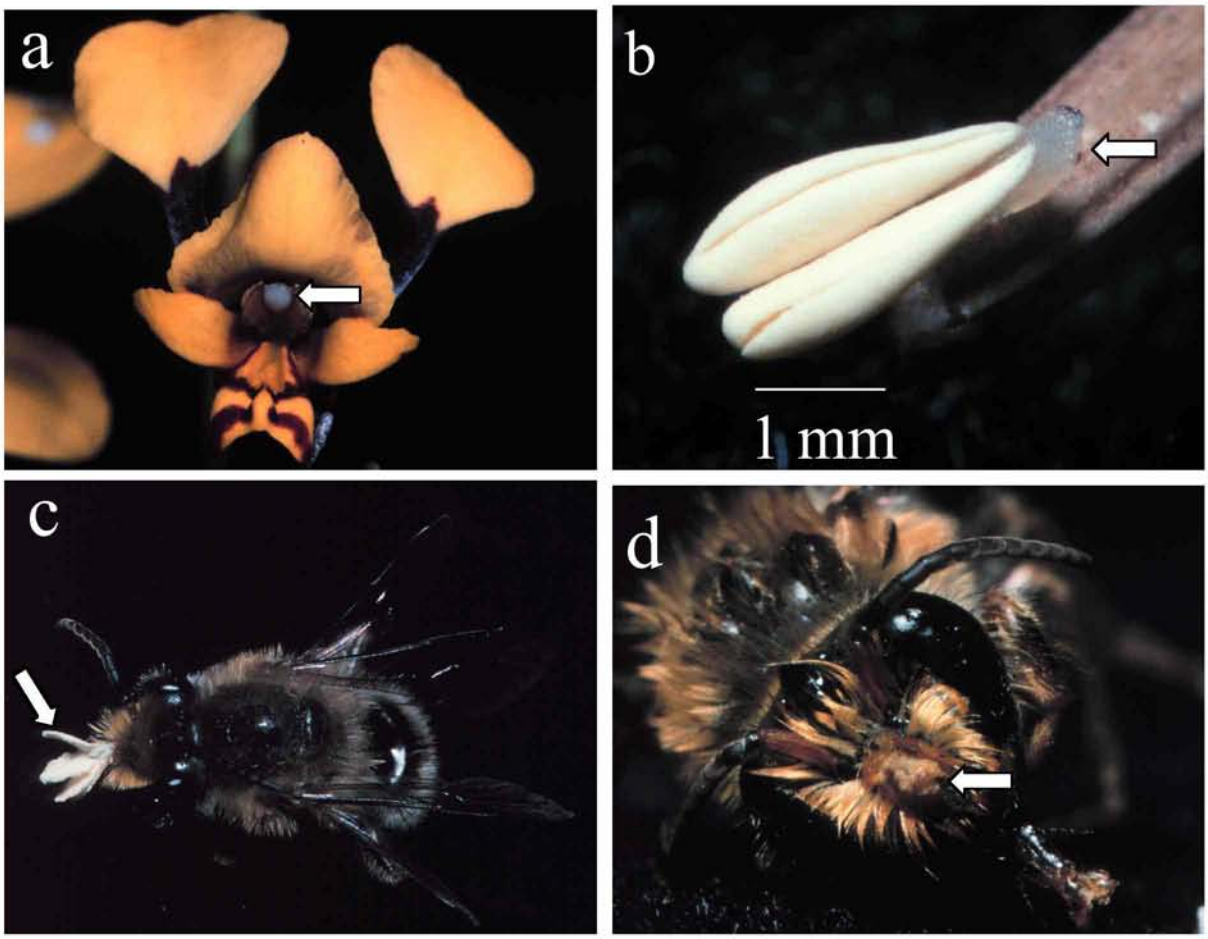

Figure 2.1. Illustrations of sources of orchid pollinaria used in this study. (a) Flower of the orchid *Diuris maculata,* which is ~ 20 mm across. The viscidium, a white sticky pad and part of the pollinarium is indicated by the arrow. (b) A pollinarium attached to a straw. The viscidium is indicated by an arrow. (c) A male *Trichocolletes venustus* bee (~8 mm in length) with orchid pollinarium (indicated by arrow) attached to its head. (d) One of four male *T. venustus* bees with orchid pollinaria remnants (indicated by arrow).

Results and Discussion

AFLP Simulation Experiment Using Varying DNA Template Amounts

Pre-selective PCR, with starting template amounts ranging from 100 ng to 6.4 pg (~15,500-fold range), produced effective amplification, as evidenced by DNA product smears, for both species of *Diuris* (Fig 2.2). Clearly visible pre-selective amplification results were obtained for template concentrations from 100 ng to 800 pg (lanes 5-9 for *D. alba* and lanes 13-17 for *D. maculata*), but there was a decline in PCR product yield progressively below 800 pg. Some product is still visible even at the lowest DNA template amount (6.4 pg; lane 12 for *D. alba* and lane 20 for *D. maculata*).

We used the same touchdown PCR successfully with both pre-selective and selective AFLP PCR reactions. The use of a higher than standard number of preselective PCR cycles with low starting DNA template produces a stronger product smear (data not shown).

Selective PCR was carried out on 20-fold dilutions of pre-selective PCR products using the primer combination 5'-HEX-*Eco*R1-AC with *Mse*1-CT. Figure 2.3 shows resulting AFLP profiles obtained with starting pre-selective PCR template DNA concentrations of 100 ng, 2 ng and 6.4 pg respectively for each of the two *Diuris* species. Figure 2.3*b*, *e* shows results for *D. maculata* and *D. alba* respectively using template at conventional concentration for pre-selective PCR and shows the characteristic pattern of DNA peaks in the size range of ~90-320 base pairs, numbered according to increasing size. No variation in AFLP profile has ever been found within either species, multiple individuals of which were samples from leaf, or pollen DNA

(>5 each of *D. maculata* and *D. alba*). However, AFLP profiles for *D. maculata* and *D. alba* can be readily distinguished as in *D. alba,* peak 3 is missing and additional peaks designated A through F are evident. Figure 2.3*a*, *d* shows successful results using 100 ng starting pre- selective PCR template was used for *D. maculata* and *D. alba* respectively. These profiles show tolerance of higher than optimal pre-selective PCR starting template. Figure 2.3*c*, *f* shows successful amplification for *D. maculata* and *D. alba* respectively with just 6.4 pg starting pre-selective PCR template. PCR product quality is clearly compromised and the relative yields of products show instability. This is probably because the starting DNA template is so dilute that the relative molarity of starting DNA is affected. Numerous spurious peaks are evident. Whilst this may be simply poor signal-to-noise ratio, it is possible that some star activity from *Eco*R1 digestion may be a contributing factor where enzyme is in gross excess (New England BioLabs Inc. 2002-03 Catalogue and Technical Reference p. 245). Importantly, within the context of this analysis, and by reference to appropriate species standards (which should be run on the same gel), the species identification always remains possible.

AFLP from Orchid Pollinaria and Pollinaria Remnants

Successful AFLP results can be obtained from orchid pollinaria removed from fresh flowers, and more or less intact orchid pollinaria removed from bees (data not shown) and the efficacy of AFLP for such samples is probably comparable to alternative procedures such as direct PCR for ITS1 (Widmer *et al.* 2000). AFLP does offer an advantage in that a "fingerprint" is the final result that with experience is instantly recognisable as belonging to a particular species. Comparable sequence data requires a certain amount of analysis with specialised computer software before the sample identity becomes clear. AFLP profiles for the more challenging samples of pollinaria

remnants from four sampled bees are shown in Figure 2.4 for the AFLP selective primer combination 5'-HEX-*Eco*R1-AC with *Mse*1-CT. AFLP profiles, although of compromised quality, match the expected AFLP profile for *Diuris maculata* (Fig. 2.3*b*) and show peaks 1-7 characteristic for this species. Confirmation was also independently obtained by using two additional primer combinations: 5'-HEX-*Eco*R1-AA with *Mse*1-CT and 5'-HEX-*Eco*R1-AA with *Mse*1-CG (data not shown).

Figure 2.4a shows the AFLP profile for the smallest and probably most degraded of the four samples collected from bees. An AFLP profile that is characteristic for *D. maculata* is clearly evident, although there is evidence of DNA degradation in the relatively higher yield of smaller molecular weight AFLP fragments. The profile for the fourth pollinaria remnant sample (Fig. 2.4*d*) shows considerable background noise, presumably owing to low starting template. Pollinarium fragments from the second and third pollinarium remnant samples were discoloured but contained more remnant tissue, probably with some pollen component and had probably been attached to the bees for a shorter period. AFLP profiles from these samples showed improved signal-to-noise (Fig. 2.4*b*, *c*) and showed less evidence of degradation. As the orchid pollinaria came from the heads of bees, contaminating pollen should not be significant, but could contribute to background noise.

AFLP generates a highly reproducible set of bands, of characteristic size (base pair number) and relative peak height when viewed as a chromatogram, or as a series of bands of varying intensity when run on an agarose gel. Thus, AFLP patterns can be readily identified visually as a "fingerprint" by comparison with a known reference. With experience, the taxonomic identity of such profiles generally becomes

immediately obvious, with the species (or individual) identity often distinguishable by one, or a few unique bands. Frequently, only a subset of these bands may be required to distinguish species, and the quality of the profile may be very poor and yet still unequivocally identifiable. Bands can be added to a profile without corrupting the information inherent in bands already present. It would therefore often be possible to resolve the DNA fingerprints of more than one species in a profile. This would have application in detection or confirmation of hybrids, or potentially to provide a species breakdown of tissue mixtures such as pollen samples from insects, which might be difficult to distinguish by microscopy.

DNA sequence data rely on faithful reproduction of an extended series of DNA bases. It is generally difficult to recognize DNA sequence data by simple inspection as characteristic for a given species and computer software is normally required to perform comparisons. In cases of "dirty" sequences there may be many ambiguous bases that confound clear identifications. In other words, a poor AFLP profile may prove more useful than poor sequence data with difficult samples. Furthermore, a possible sample of mixed species source, with a mixed AFLP profile is likely to be recognised as such more readily than such a sample in the form of "dirty" DNA sequence.

Unlike most PCR-based analysis of DNA, the AFLP primer design of Vos *et al.* (1995) is not based on genomic sequence, but is based on potentially optimal DNA adapters. Certainly, the remarkable capacity of AFLP amplifications to succeed with very low starting template suggests highly successful adapter and primer design. The use of a two-step PCR approach is also an advantage. One factor is that initial use of unmodified primers maximises robustness in the first critical PCR cycles. Any primer

modification, whether a radiolabel, or a fluorescent tag, is likely to have some effect on the PCR robustness, so the unlabelled preselective primer is likely to minimise PCR failure (Indsto *et al.* 2001). High BSA concentration of up to 1 µg/µl in the PCR (Giambernardi *et al.*1998) overcomes melanin inhibition and probably other PCR inhibitors as well as stabilising the *Taq* DNA polymerase. The use of 2% formamide in the PCR reactions (Ranamukhaarachchi 2000) is useful in minimising spurious amplification whilst also improving signal intensity.

It is possible to run AFLP samples on agarose gels, or similar higher resolution equivalents. Whilst in some cases this may provide all the information necessary, higher resolution, to one-base separation, can be obtained using denaturing PAGE. The use of fluorescent labels on one of the PCR primers and laser detection combines the best of sensitivity and resolution, and is becoming increasingly popular. Whilst such samples are often sent to a DNA sequencing facility for analysis, alternatives exist. One such alternative is the Corbett Gel-Scan machines, which operate on a simplified (and much cheaper) version of similar technology to the Applied Biosystems sequencing systems.

In summary, Vos et al. (1995), showed, in principle, the forensic potential of AFLP by the successful amplification of DNA of very low concentration, but stopped short of demonstrating a forensic application. At the present time AFLP would not be regarded by many researchers as a viable analytical tool for forensic sample identifications. However, in this study we show that not only is this possible, but the method may show advantages over alternative approaches, particularly for difficult samples containing very low quantities of DNA.

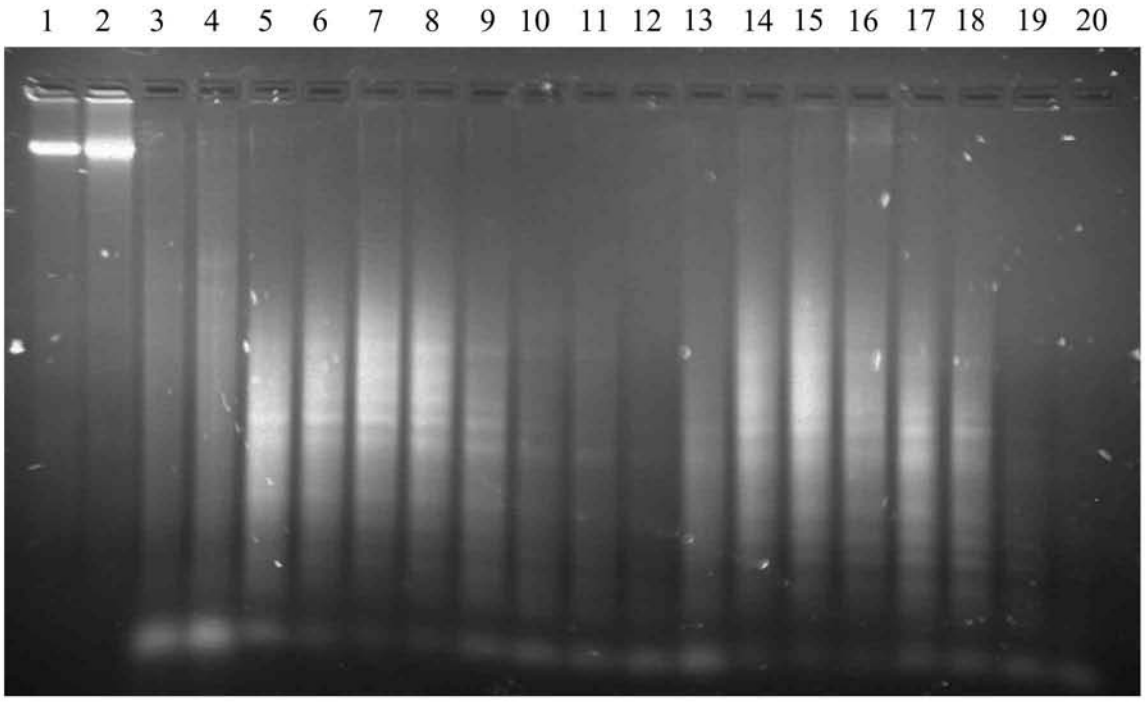

Figure 2.2. Agarose gel showing genomic DNA and pre-selective PCR products. (Lane 1) 250 ng *Diuris alba* and (Lane 2) 250 ng *Diuris maculata* genomic DNA. (Lanes 3, 4) 250 ng *D. alba* and *D. maculata* genomic DNA respectively after restriction/ligation. Complete digestion is indicated by a smear of multiple DNA fragments with no high molecular weight band visible. (Lanes 5-12) 10 µL *D. alba* pre-selective PCR products with starting DNA templates ranging from 100, 50, 20, 4 ng and 800, 160, 32 and 6.4 pg respectively. A range of PCR product sizes produces a visible smear. (Lanes 13-20) 10 µL *D. maculata* pre-selective PCR products with the above starting DNA template concentrations and showing similar pre-selective PCR product smears.

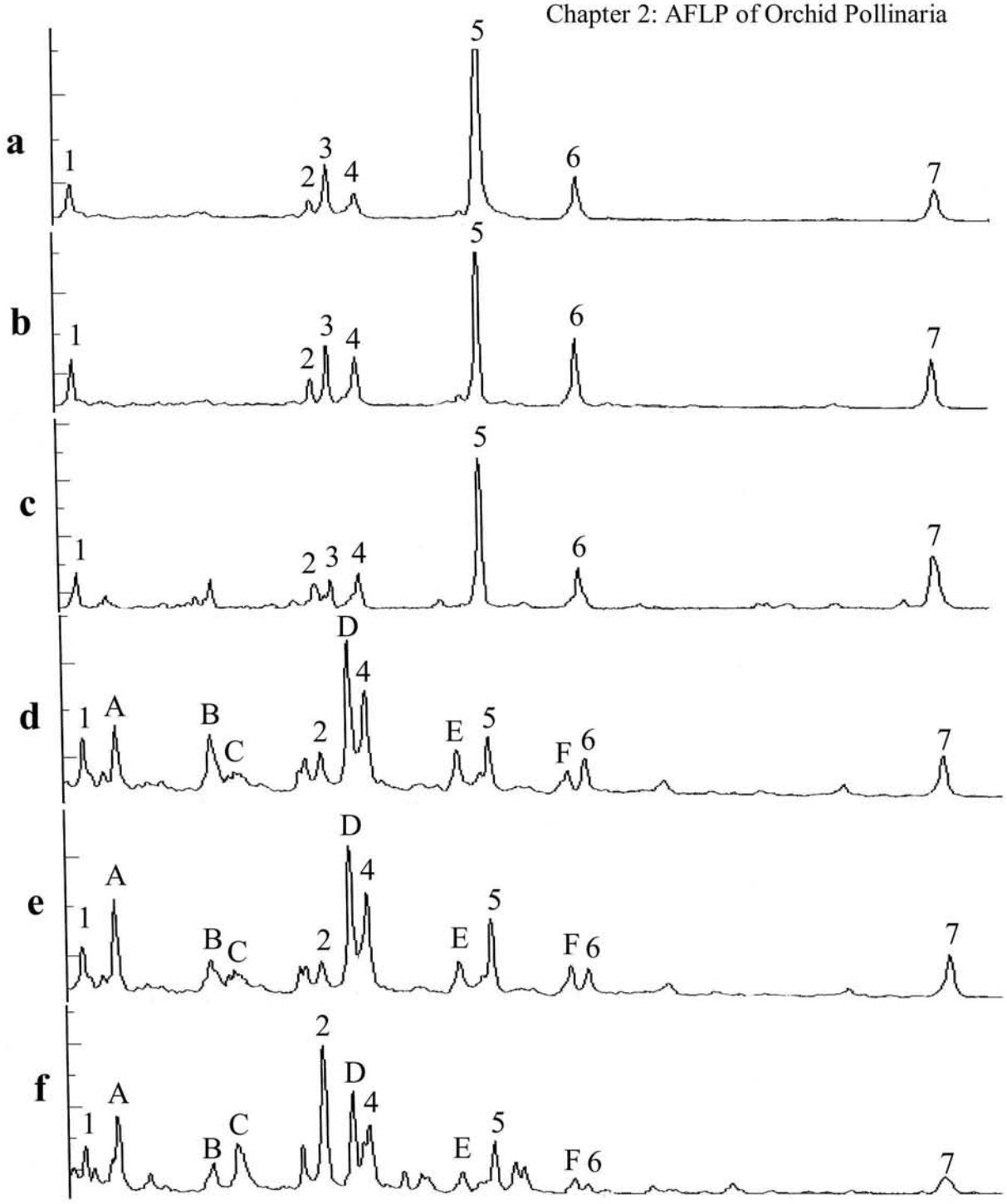

Figure 2.3. AFLP simulation experiment results with the selective primer combination 5'-HEX-*Eco*R1-AC with *Mse*1-CT. Profiles for *D. maculata* from (*a*) 100 ng, (*b*) 2 ng and (*c*) 6.4 pg starting DNA pre-selective PCR product (*d-f*) as above, but for the species *D. alba*. Note that profiles for 6.4 pg starting DNA pre-selective PCR products give a final selective PCR product with disturbed product ratios of bands and increased signal noise. See Results section for further details.

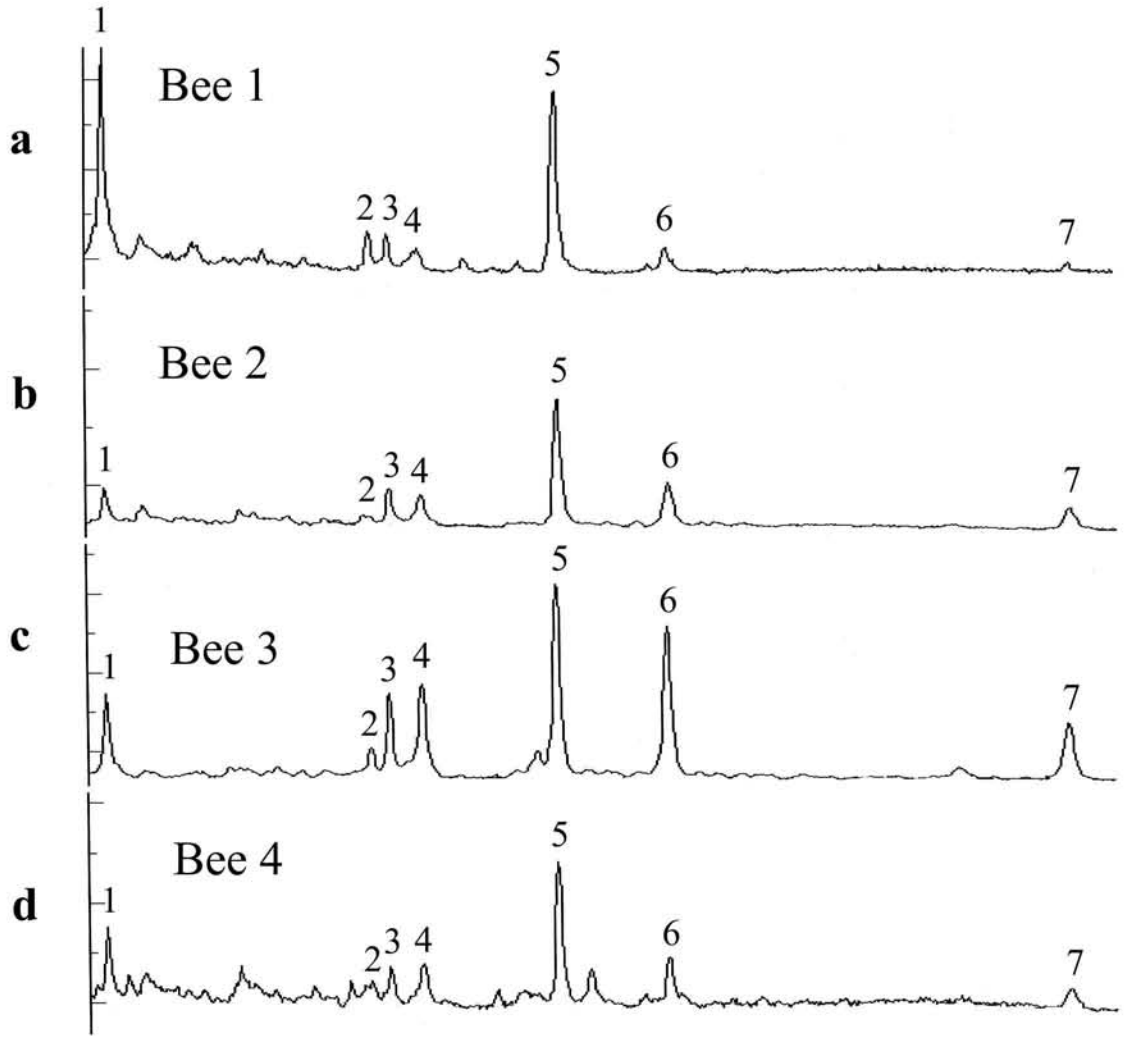

Figure 2.4. AFLP results with the selective primer combination 5'-HEX-*Eco*R1-AC with *Mse*1-CT from DNA extracts of orchid pollinaria remnants. (*a-d*) AFLP profiles for bees 1-4 respectively. Note that all show the same basic profile which matches that expected for *D. maculata*. See Results section for further details.

Acknowledgements

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Chapter 3

Pollination of *Diuris maculata* (Orchidaceae) by male *Trichocolletes venustus* bees

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Prologue

This work has been published as a research paper (Indsto, JO, Weston, PH, Clements, MA, Batley, M, Whelan RJ (2006) Pollination of *Diuris maculata* (Orchidaceae) by male *Trichocolletes venustus* bees. *Australian Journal of Botany* **54**: 669-679). Peter Weston, Mark Clements and Rob Whelan provided guidance on project design. Andrew Perkins showed me the site in Scheyville National Park of the *Diuris maculata* colony. Mark Clements provided much useful information on *Diuris maculata* colonies and flowering behaviour. Michael Batley gave me considerable advice on native bee biology and identified native bees. Reflectance spectrophotometry and colorimetric analysis of flowers was carried by Adrian Dyer. Rob Whelan and Peter Weston provided assistance in getting the manuscript into publishable form.

Abstract

In a previous study, the Australian terrestrial orchid *Diuris maculata sensu lato* from a site in near Melbourne in Victoria, was suggested to be a floral mimic of several sympatric legume species. The widespread distribution of this orchid (or species complex) suggests that there may be a number of different model and pollinator species throughout this range, and that additional studies are necessary to characterise its pollination adequately. In this study, the pollination of *D. maculata* in the Sydney region, mainly at Scheyville National Park, was compared with the results previously obtained in Victoria. At Scheyville National Park, *Trichocolletes venustus* was the only native bee species found in significant numbers, and the flowers it visited were almost

exclusively the legumes *Hardenbergia violacea* and *Daviesia ulicifolia* ssp. *ulicifolia*. Fifty percent (14 of 28) of captured male bees carried *D. maculata* orchid pollinaria, or remnants, which were identified using AFLP fingerprinting. Female bees, which appeared about 10-14 days after males, were not observed visiting the orchid, or carrying orchid pollinaria. We confirm *D. maculata* flowers lack nectar, and note the pea-like flowers possess an ultraviolet false nectar guide comparable to the true ultraviolet nectar guide of the legume flowers. Colorimetric analysis showed the colour separation between *Daviesia ulicifolia* ssp. *ulicifolia* and the orchid is small enough to produce foraging errors, consistent with mimicry. We conclude that guild mimicry of "egg and bacon" legumes best explains the pollination of *D. maculata* s.l., rather than precise mimicry of any one pea species. Preliminary observations suggest that pea-flower mimicry may range from being highly precise in some species, through to being much more generalised, but still retaining elements of mimicry. The novel finding of comparable UV patterns in *Diuris* species and putative pea models applies to most species in the genus and we found that the rare *D. aequalis* shows remarkable similarity in colour, shape and ultraviolet patterns to the sympatric legume *Gompholobium huegelii*, and is likely to be a mimic of this species.

Introduction

Batesian mimicry is traditionally defined in predator-prey coevolution as resemblance of an innocuous species to one that is distasteful to predators. This process was first proposed by Henry Bates, who discovered this form of mimicry in butterflies during his

11 years in the Amazon (Bates 1862). However, this term has also been more broadly applied by some authors to any relationship in which a mimic (e.g. a palatable species) elicits, and benefits from, the same behavioral response (e.g. avoidance) from an operator (e.g. a predator) as does the model that it resembles (e.g. a distasteful species). This broader concept has been applied to the relationship between many orchid species (mimics), their animal pollinators (operators) and plants or animals that they resemble (models) (Cingel 2001). For example, flowers of the orchid *Chiloglottis trapeziformis* (mimic) resemble females of the thynnine wasp *Neozeleboria cryptoides* (the model) so closely in scent and appearance that males of *N. cryptoides* (the operator) attempt to mate with them, pollinating them in the process (Schiestl *et al.* 2003). Sexual deception of this kind, in which the operator gains no benefit from the mimic, has been documented in numerous species of at least ten genera of orchids (Pridgeon *et al.* 2001).

Another subclass of Batesian mimicry consists of plant species with flowers that closely resemble the floral food sources (models) of their pollinators (operators). Although floral food source mimicry is thought to be widespread in the Orchidaceae (Cingel 2001; Pridgeon *et al.* 2001), well-documented examples are relatively scarce. Published accounts generally propose a specific model and pollinator (Kjellsson *et al.*1985; Johnson 1994; Johnson and Edwards 2000; Sugiura *et al.* 2002), but Dafni and Ivri (1981) proposed a Batesian mimicry system for *Orchis israelitica* on a lily, *Bellevalia flexuosa*, involving a number of pollinators. In most of the documented cases the orchid shows a remarkable visual similarity to the putative model, but Sugiura *et al.* (2002) in a study of the pollination of *Cypripedium macranthos* var. *rebunense* by a bumble bee, noted that this orchid shows colour similarity, but not floral form similarity, to *Pedicularis schistostegia* (Orobanchaceae). They cited field experiments

(Wilson and Stine 1996) that show that bumblebees visit flowers of similar colour and different shapes, but not *vice versa*.

In a review of floral mimicry, Roy and Widmer (1999) pointed out that few studies, other than Dafni and Ivri (1981) and Johnson (1994), addressed the issue of whether the orchid mimic shows greater reproductive success in the presence of the model. Sugiura *et al.* (2002) in their more recent study, however, show that *Cypripedium macranthos* var. *rebunense* has increases pollination frequency when growing near the model species. Despite such evidence, testing the hypothesis of Batesian food source mimicry in orchid pollination is problematic. Putative Batesian mimics showing greater fecundity in the presence of the model may do so simply because pollinators aggregate where food is present (see discussion by Johnson (1994)). Distinguishing between such possible incidental benefit and true mimicry would be a challenge. The characteristically low fecundity of orchid Batesian mimics compared to rewarding species is also difficult to explain in evolutionary terms of selective benefit. Batesian mimicry is likely to result in greater outcrossing and reduced pollen wastage, an important factor for an orchid because all the pollen, in the form of a pollinarium, is contained in one parcel. As the mimic relies on deception for pollination, pollinators would be expected to learn to avoid it if it was frequently encountered, leading to rarity of the mimic relative to the model (Dafni and Bernhardt 1990).

The study by Beardsell *et al.* (1986) of the pollination of *Diuris maculata s.l.*, at a site near Melbourne, Victoria provided a major advance from the largely anecdotal observations recorded previously. In particular, their study utilised the majority of the relevant data that a serious pollination study of its time could employ to test the

hypothesis that this orchid may be a food-source mimic of 'egg and bacon peas' belonging to the genera *Daviesia*, *Pultenaea* and *Dillwynia*. However, it is clear that the above study of one taxon at one site could only be the beginning of the necessary research to establish not only the fundamentals of the pollination biology of this widespread species, or species complex, but of a body of evidence that could provide insight into the pollination of the whole genus. We therefore extended this first, groundbreaking study by testing the hypothesis that *D. maculata* (or closely related taxon) in the Sydney region might have a fundamentally similar mode of pollination, despite expected differences in putative model and pollinator species. Our study, some 20 years later than the original study by Beardsell *et al.* (1986), might be expected to employ some additional lines of evidence currently available. Thus, we include data from ultraviolet imaging of orchid and putative model flower flowers, DNA fingerprinting of orchid pollinaria and pollinaria remnants by AFLP, colorimetric analysis of flowers to test the hypothesis that this orchid mimics the colour of model pea flowers in the insect visual system, and nectar sampling.

Materials and Methods

Diuris maculata s.l. is a widespread species complex, extending from at least as far north as Taree (31° 54′S, 152°27′E) through eastern New South Wales and much of Victoria, to Tasmania and South Australia, as far west as the Eyre Peninsula (Bishop 2000). It is associated with woodland habitat that has a shrubby understorey dominated by legume shrubs of several genera, particularly *Dillwynia*, *Pultenaea* and *Daviesia*. The morphological similarity of these populations and similarities in habitat preferences led us to propose the hypothesis that they share a functionally similar pollination

system. The forest communities, legume model species and pollinators are likely to vary considerably over such a wide distribution. It follows that pollination of the *D. maculata* species complex can hardly be understood from study at just one site. Additional studies are therefore necessary to test the hypothesis that the mode of pollination mode remains constant despite changes in the specific model and pollinator species. We undertook this study of the pollination of *D. maculata* in the Sydney region, approximately 1000 km from the original study site in Victoria described by Beardsell *et al.* (1986). We worked mainly in a population at Scheyville National Park (33°35´S, 150°52´E), but with supplementary data from a population at Pennant Hills (33°44´S, 151°03´E), a flowering stem of which is illustrated in Figure 3.1.

Figure 3.1. *Diuris maculata* from Stringy Bark Ridge, Pennant Hills, in northern Sydney, New South Wales. Flowers are about 20 mm across.

The study population, in Scheyville National Park near Pitt Town in western Sydney, is on a gently sloping site in dry sclerophyll forest dominated by a stand of *Eucalyptus fibrosa* (Myrtaceae). Scattered shrubs included *Hakea sericea* (Proteaceae), *Olearia* sp. (Asteraceae) and the legume *Daviesia ulicifolia* ssp. *ulicifolia* (Fabaceae: Mirbelieae), although nearby this formed a dense thicket. The shrub *Lissanthe strigosa* ssp. *subulata* (Ericaceae) was also scattered, but formed patches nearby. The ground cover comprised *Themeda australis* (Poaceae) with the purple-flowered climbing legume *Hardenbergia violacea* (Fabaceae: Phaseoleae). The soil is a reddish clay derived from Wiannamatta shale, which is overlaid in places with a lighter pale brown soil, presumably of alluvial origin. *Diuris maculata* was restricted to areas where the alluvium achieved a depth of about 10 cm or more.

The study of the pollination of *Diuris maculata* was conducted across three flowering seasons, 2001, 2002 and 2003, all of which were somewhat drought-affected. Plants were confined mainly to a narrow strip within 3m of a track running about 12 m through the site. In 2001, the least drought-affected year, about 100 flowering plants were seen, but in 2002/3, only about half this number flowered. *D. maculata* typically begins to flower about the first week in August when few other flowers are open; these include the first flowers of *Hardenbergia violacea* and *Lissanthe strigosa* ssp. *subulata*. Peak flowering of the orchid is usually mid-August, which coincides with the main flowering of *Hardenbergia violacea* and the first flowers of *Daviesia ulicifolia* ssp. *ulicifolia*. The main flowering for this latter shrub is not until the end of August, when *D. maculata* is finishing. *Diuris maculata* plants at this site showed a rather general

similarity to yellow-flowered peas (*Daviesia ulicifolia* spp. *ulicifolia*) at this site. *D. maculata* flowers were not found to have noticeable fragrance.

Pollination statistics

A pollinarium in *Diuris* comprises the two pollinia plus the viscidium, the sticky pad that attaches to the face of an insect. We used pollinarium removal as a measure of successful visitation by pollinators, since accidental removal by other means can be expected to be rare. Fruit set was determined for 2001 and 2003 when sufficient moisture extended into September to permit fruit development. Data for 2001, from 63 plants were collected in the cumulative manner involving total inflorescences in a site as per Beardsell *et al.* (1986). Instead it was found that a more simplified approach could adequately summarise the key pollination features and so data were collected for pollinaria removal at advanced peak flowering (which provided a slight underestimate of total pollinaria removal), followed by measurement of fruit set *v.* total flowers of marked inflorescences some 4-6 weeks after flowering had finished.

In addition to the study of a *D. maculata* population at Scheyville National Park, a second population was found by one of us (J.I.) at Stringybark Ridge, Pennant Hills on 24 August, 2003 at an advanced stage of flowering. Data from this population was used to supplement the main body of data from Scheyville National Park.

Pollinator sampling

Captured putative pollinating insects were killed by being held in the net over dry ice.

Insects were stored frozen at -20°C in numbered microfuge tubes. Representative samples of pollinators have been lodged with the Australian Museum, Sydney.

Pollen analysis

Pollen was washed from bodies of all captured insects with 70% ethanol onto uncharged microscope slides and, upon drying, stained with Calberla's fluid (Ogden et al. 1974) before the addition of a coverslip, which was sealed with clear nail varnish. Reference samples of pollen were taken from voucher specimens of sympatric flowering plants and *D. maculata*.

DNA analysis of pollinaria and remnants using AFLP

Pollinaria and most of the remnants removed from captured bees were DNA fingerprinted using AFLP to confirm the tissue source using a modified AFLP protocol (Indsto *et al.* 2005).

Testing of visual cues using ultraviolet reflectance photography and colorimetric analysis

Australian pea flowers of the tribe Mirbelieae such as species of *Dillwynia* and *Pultenaea* are known to have an ultraviolet colour component and ultraviolet-absorbing nectar guides, which are perceived by hymenopteran floral visitors (Kay 1987). Consequently, *D. maculata*, which putatively mimics flowers of these pea species, was studied for the presence of comparable patterns, consistent with mimicry. Ultraviolet reflectance photography was carried out on flowers of *D. maculata s.l.* and putative

model flowers as described elsewhere (Williams and Williams 1993; Indsto and Weston 1999), by using patches which reflect evenly throughout the visible and near-UV range. These permit brightness comparison of visible and ultraviolet images (Kevan 1979; Dyer 1996). Colorimetric analysis of orchid and putative model flowers (Backhaus 1991; Chittka 1992) was undertaken to test whether putative mimic flower colours could lead to colour-based foraging errors by pollinators (Dyer and Chittka 2004). In this study, we sought to test whether the yellow flowers of the orchid were sufficiently similar, in the insect visual system, to the yellow flowers of the pea *D. ulicifolia* spp. *ulicifolia* for colour-based foraging errors to occur. As we note below, the purple flowers of the pea *H. violacea* were also visited by pollinating bees. In this case the colours of the pea and orchid were expected to be rather dissimilar and we therefore included data to test this expectation. Numerous other sympatric flowering species are highly dissimilar in colour to the orchid and we show this with the example of the sympatric white-flowered daisy *Olearia* sp. Reflectance spectrophotometry of flowers in the bee's visual range of 300- 650 nm was undertaken and the data were analysed according to the sensitivities of bee's colour photoreceptors. The colour of outer floral parts was sampled by removing these from a number of flowers, from different plants, and arranging them in a tiling manner across a sampling disc. The resulting colour measurement was therefore an averaged measure of colour for each species. Spectral reflectance of plant flowers and foliage was measured with a Varian DMS100 reflectance spectrophotometer calibrated against a Varian polytetrafluoroethylene standard. The colour loci of flowers was calculated in a hexagon colour space (Chittka 1992) considering the spectral sensitivity functions of honeybee photoreceptors (Menzel and Backhaus 1991). Although the spectral sensitivity of Australian native bees has not yet been measured, the spectral sensitivity of honeybee photoreceptors can be taken to

be representative of trichromatic visual capabilities of most hymenopteran insects (Kevan *et al.* 2001).

The relative amount of light absorbed by each photoreceptor class is given by *P*:

$$P = R \int_{300}^{650} Si(\lambda)I(\lambda)D(\lambda)d\lambda \qquad (1)$$

Where $Si(\lambda)$ is the spectral sensitivity of the (UV, blue, green) receptor classes, $I(\lambda)$ is the spectral reflectance function of the stimulus and $D(\lambda)$ is the spectral distribution of the illuminant. The variable R simulates adaptation to the painted green background (I_B),

$$R = 1/\int_{300}^{650} S(\lambda)I_B(\lambda)D(\lambda)d\lambda \qquad (2)$$

The transduction of photoreceptor absorption (P) into receptor excitations (E) is given by

$$E = P/(P+1) \qquad (3)$$

Coding is assumed to be performed by two unspecified opponent mechanisms and colour distance can be calculated as the Euclidean distance between colour loci (Chittka 1992).

Nectar sampling

In order to determine whether very small amounts of nectar were present, a method similar to that of Gross (1990) was used, in which 2 μL of distilled water was pipetted into the flower spur, or the base of the stigma in the case of orchid flowers, using a Gilson P20 micropipette with a drawn-out tip as used for loading DNA sequencing gels (e.g. the Applied Biosystems ABI Prism 377 DNA Sequencer). The liquid was pipetted up and down several times and tested for sugars using a Bellingham and Stanley Eclipse 45-81 refractometer adapted for low volumes. Sugar content (of diluent) as low as 0.5% could readily be detected after calibration with distilled water.

Results

Putative pollinators

The only Australian native bee found at the site in significant numbers was *Trichocolletes venustus*. Occasionally small native bees such as *Exoneura* sp. were found on legumes, but did not appear involved in orchid pollination. In 2001 and 2002, the introduced honeybee, *Apis mellifera* (Hymenoptera: Apidae), was very common and outnumbered native bees about 10:1, but in the spring of 2003 this bee was virtually absent. As the summer of 2002/3 was very hot and dry, it is possible that local honeybee nests collapsed from stress. Other common flower visitors included small butterflies and hoverflies, but neither of these seemed capable of pollinating *D. maculata*.

Visits of insects to *D. maculata* were infrequent and only four visits were noted in 2001 over more than 8 h of observation. On two occasions in 2001 a male *T. venustus* was seen to fly from *Hardenbergia violacea* to *D. maculata* inflorescences about 1-2 m

away. Visits were very brief with several plants being visited in succession over about 5 s. Similar behaviour was observed from honeybees. However, on the two occasions honeybees were seen to visit *D. maculata* they were seen to drop *D. maculata* pollinaria, suggesting that they do not adhere very well to their faces. No *Apis mellifera* captured bore orchid pollinaria.

Bee sampling

Bees were sampled in 2001 and 2002, with most being sampled around peak flowering time for *D. maculata*. In all, 28 male *T. venustus* plus 8 female *T. venustus* were sampled. Females were more abundant at the end of the orchid flowering period, which corresponded to the main flowering of *D. ulicifolia* ssp. *ulicifolia*. Of the 28 male bees, two had obvious orchid pollinaria on the face, which was confirmed to be *D. maculata* using AFLP fingerprinting. One of these bees is shown in Fig. 3.2*a*. Additionally, on close examination with a 10X hand lens, it was clear that a further 12 male bees had pollinaria remnants, often comprising little more than a dessicated viscidium. The viscidium material, which was remarkably persistent, was removed from 7 of the bees and found to be from *D. maculata* using a modified AFLP protocol (Indsto *et al.* 2005). Therefore, it can be shown that 50% (14/28) male *Trichocolletes venustus* bees had at some stage collected pollinaria of *D. maculata*. No female bees carried orchid pollinaria or remnants. No honeybees were seen with orchid pollinaria or remnants, this being confirmed by capture and release of about 25 bees and careful examination of many others whilst foraging.

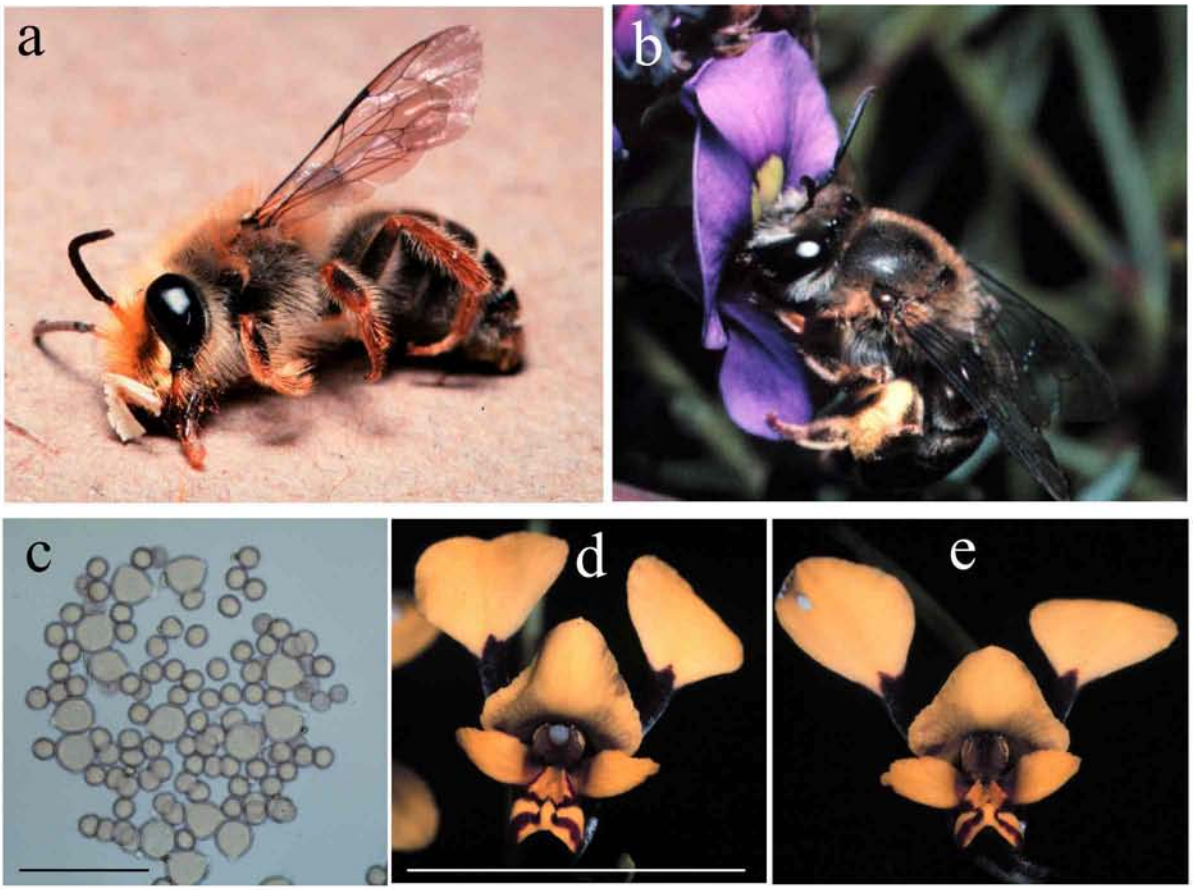

Figure 3.2. A montage showing (*a*) a male *Trichocolletes venustus* bee caught with *Diuris maculata* pollinia attached to the face (males of this species are about 8 mm long). (*b*) A female *T. venustus* bee (slightly larger than males) foraging at a flower of *Hardenbergia violacea*. Note that the bee is probing for nectar at the base of the nectar guide whilst working the flower keel for pollen with its legs. Note: Male and female bees of this species can be readily identified in the field based on size and the hairier faces of the males. (*c*) Pollen washed from a female *T. venustus* bee caught foraging at *H. violacea*. Two types of legume pollen are present. The larger, more triangular grains are from *H. violacea* and the smaller, rounder grains are from *Daviesia ulicifolia* ssp. *ulicifolia*. The scale bar = 100 µm. A *D. maculata* flower with pollinarium (*d*) present and (*e*) removed. The scale bar = 2 cm.

Flowering phenology and pollination statistics

In 2001, flowering of *D. maculata* began about the beginning of the fourth week of July, peaked around 12 August, and extended to the first few days of September. In 2002 and 2003, apparently due to cooler and drier conditions, flowering was delayed for about two weeks. Table 3.1 shows a summary of pollination statistics for 2001. Approximately 20% of flowers or buds were either damaged or aborted prematurely. Small case moth caterpillars appeared the most common herbivore. Pollinaria removal is the cumulative total. Figure 3.2*d* shows a flower of *D. maculata* with the pollinarium present. The viscidium can be seen as a white disc at the flower centre. Fig. 3.2*e* shows the same flower with the pollinarium removed. Fruit set (green and obviously swollen ovaries) was counted about 4 weeks after flowering finished on 29 September 2001.

Table 3.1. Summary of pollination statistics for 63 plants of *Diuris maculata* for 2001 at Scheyville National Park. Percentages are given in parentheses

Total flowering plants (or clumps)[A]	Total inflorescences	Total flowers + buds	Pollinaria removed/total flowers + buds
63	75	294	101/294 (34)

Fruit set/ total flowers + buds	Inflorescences bearing fruit/total	Inflorescences bearing > 1 fruit/ inflorescences bearing fruit
37/294 (12.5)	24/75 (32)	10/24 (42)

[A]The orchid occurs mostly as single growths with one inflorescence. However, large plants with multiple growths and multiple inflorescences are not uncommon.

Few pollination data are available for 2002 because of particularly dry conditions in this year. However, a check for pollinaria removal when the orchids were at mid-late flowering stage (16 plants with 73 flowers all open plus 9 finished) at the end of August showed that 38/73 (52%) of flowers had had pollinaria removed. Very little rain over the following 6 weeks would have greatly reduced fruit development, so it was not considered worthwhile to collect these data. In 2003, only data for fruit set were

recorded, on 20 September 2003, for 32 plants. Fifteen of 32 plants produced fruit with 5/15 (33%) having more than one fruit. Of 125 flowers, 20 (16%) had set fruit.

Supplementary pollination data from Stringybark Ridge, Pennant Hills were also obtained. Pollinaria removal was found to be high at an advanced stage of flowering on the 24 August 2003. Thirty plants checked had 56 of 88 (64%) of flowers with pollinaria removed, plus 39 flowers finished. Fruit set was checked on 19 September 2003, about 3 weeks after flowering finished. In total, 17 of 29 plants (59%) had set fruit with 3/17 (18%) having more than one fruit. Of 122 flowers, 21 (17%) had set fruit. These pollination statistics from two sites, one of which was sampled across 3 years, can be summarized to a simple consistent outcome as follows: each year about one half to two-thirds of flowers for this orchid species have their pollinaria removed, but fruit set was consistently much lower and within a fairly small range of 12.5-17%. These results also match closely those reported for Beardsell *et al.* (1986) at their study site in Melbourne.

In early August, 2005 the Pennant Hills population of *Diuris maculata* was again found to be in bloom, but with only about one-third the numbers seen in 2003. At this site, on a fine day, it was noted that few legumes were in bloom, except for two mature plants of *Dillwynia retorta* and small numbers of *Hovea linearis*. Male *T. venustus* bees were abundant, females having not yet emerged, and were visiting flowers of both these legumes species. Approximately 10% of these bees carried obvious orchid pollinaria. This observation suggests that male *T. venustus* is probably the main pollinator of *D. maculata* at this site and, we would expect, at probably many other sites at which this orchid occurs in the Sydney region, where this bee seems to be ubiquitous.

Ultraviolet imaging

Figure 3.3 shows comparative human visible range and near-ultraviolet light photographs *of D. maculata* and the sympatric legumes *H. violacea* and *D. ulicifolia* ssp. *ulicifolia* from Scheyville National Park. Figure 3.3*a* shows *D. maculata* with *D. ulicifolia* ssp. *ulicifolia* under visible light. Four reflectance patches in the photograph corners reflect from bottom left and then clockwise, 40, 30, 20 and 10% respectively, of incident light in both visible and ultraviolet wavelength ranges. Reflectance patches allow matching of brightness to visible light for UV images. It is possible otherwise to grossly over- or under-brighten UV images with spurious results (Kevan 1979). Note that both the orchid and legume reflect significantly overall in both the visible and UV ranges. Under ultraviolet light (Fig. 3.3*b*) the orchid callous ridges and the column appear dark since they lack UV reflectance and form a false nectar guide. Figure 3.3*c*, *d* shows comparative photos for the purple legume flower *H. violacea* from which it can be seen that the flowers reflect significantly overall in both visible and UV ranges. However, the two pale green spots at the base of the standard petal (Fig. 3.3*c*) under ultraviolet light (Fig. 3.3*d*) appear very dark and lack ultraviolet reflectance, forming an ultraviolet nectar guide. Figure 3.2*b* shows a female *T. venustus* bee foraging on *H. violacea*. The bee can be seen to be probing for nectar at the base of the nectar guide while collecting pollen with its legs. An enlargement *of D. ulicifolia* ssp. *ulicifolia* flowers (Fig. 3.3*e*, *f*) shows similar visual cues to *H. violacea*. In the case of this flower a clear spot is visible within a reddish crescent at the base of the standard petal under visible light (Fig. 3.3*e*), which absorbs ultraviolet light (Fig. 3.3*f*). Given that the legume flowers possess UV nectar guides visible to foraging bees, it would be expected that the orchid *D. maculata* would also possess similar features in order to function as a food-source mimic. This was confirmed by the observations.

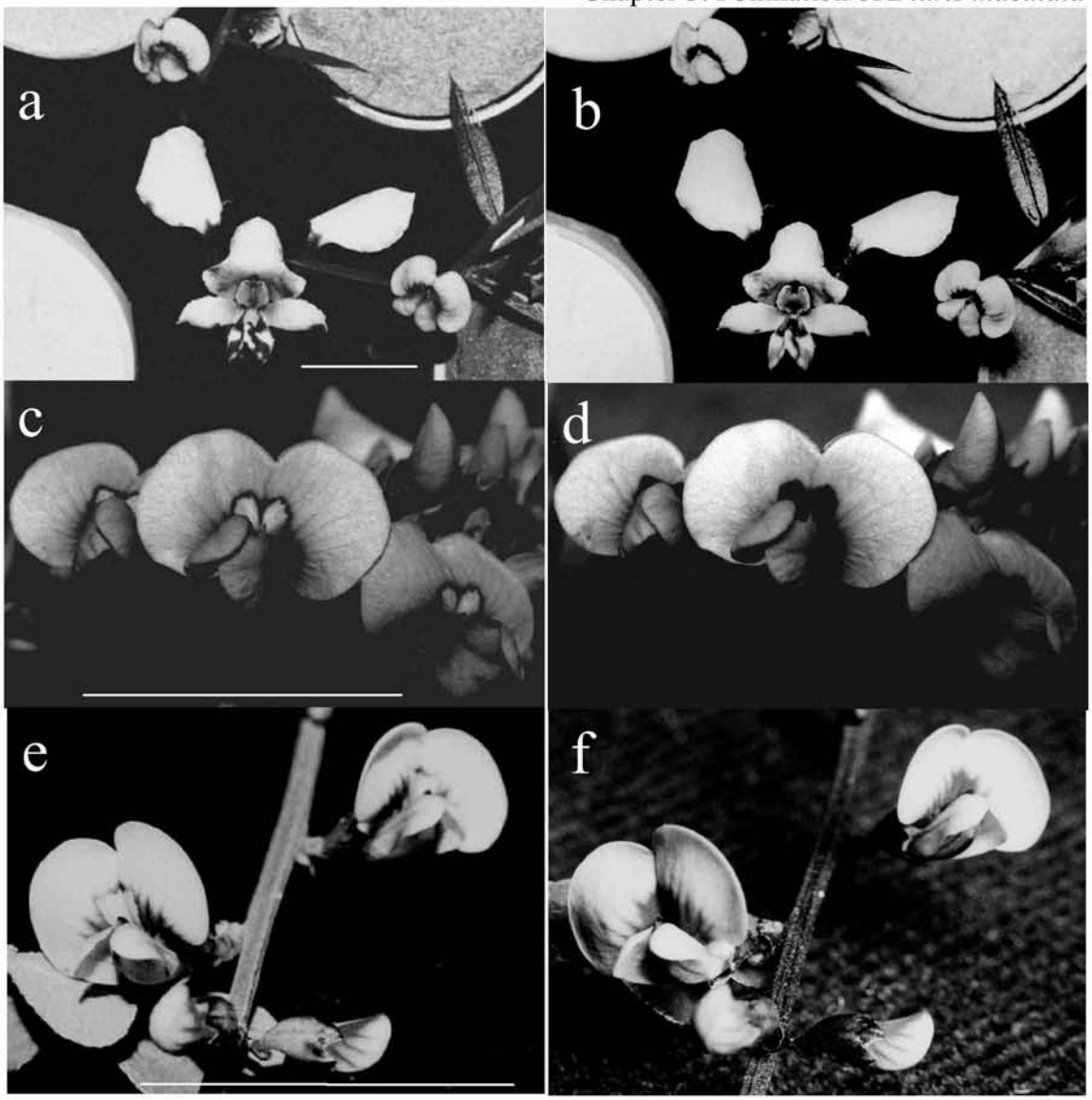

Figure 3.3. Comparative black and white photographs in the human visible range (HVR) compared with corresponding near-UV images. (*a*) HVR image of a *Diuris maculata* flower with *Daviesia ulicifolia* ssp. *ulicifolia* and (*b*) the comparative UV image. Note that both the orchid and legume flowers reflect in the UV range and that in the orchid a region comprising the labellum callous ridges and column forms an UV-absorbing false nectar guide. Note: photograph corners contain plaster reflectance patches reflecting (clockwise from bottom left) 40%, 30%, 20% and 10% of light throughout the UV and HVR range. (*c, d*) Comparative images for the legume *Hardenbergia violacea* in HVR and UV range, respectively. (c) In *H. violacea* two pale green spots occur at the base of the standard petal and (*d*) these form UV-absorbing nectar guides. (*e, f*) Comparative much-enlarged images of flowers of the legume *D. ulicifolia* ssp. *ulicifolia*. (*e*) Under HVR light a reddish crescent is found at the base of the standard petal with a clear patch. (*f*)This whole region forms an UV-absorbing nectar guide. Scale bars = 1 cm.

Colorimetric Analysis

Figure 3.4 shows flower colours for *H. violacea* (A), *D. maculata* (B) and *D. ulicifolia* ssp. *ulicifolia* (C) plotted in a colour hexagon. Dyer and Chittka (2004) experimentally studied colour-based foraging errors of bumblebees and showed that with an increasing colour distance between target and distractor flowers, fewer foraging errors were made. The flowers of *H. violacea* are well separated from *D. maculata* for the colour visual system of bees (0.22 colour hexagon units; Fig. 3.4) and would be distinguishable by a bee with almost 100% accuracy (Dyer and Chittka 2004). However, the flowers of *D. maculata* are only separated by about 0.058 colour hexagon units from *D. ulicifolia* ssp. *ulicifolia* and bees could be expected to make colour-based foraging errors about 25% of the time, a finding consistent with mimicry by *D. maculata* of *D. ulicifolia* ssp. *ulicifolia*.

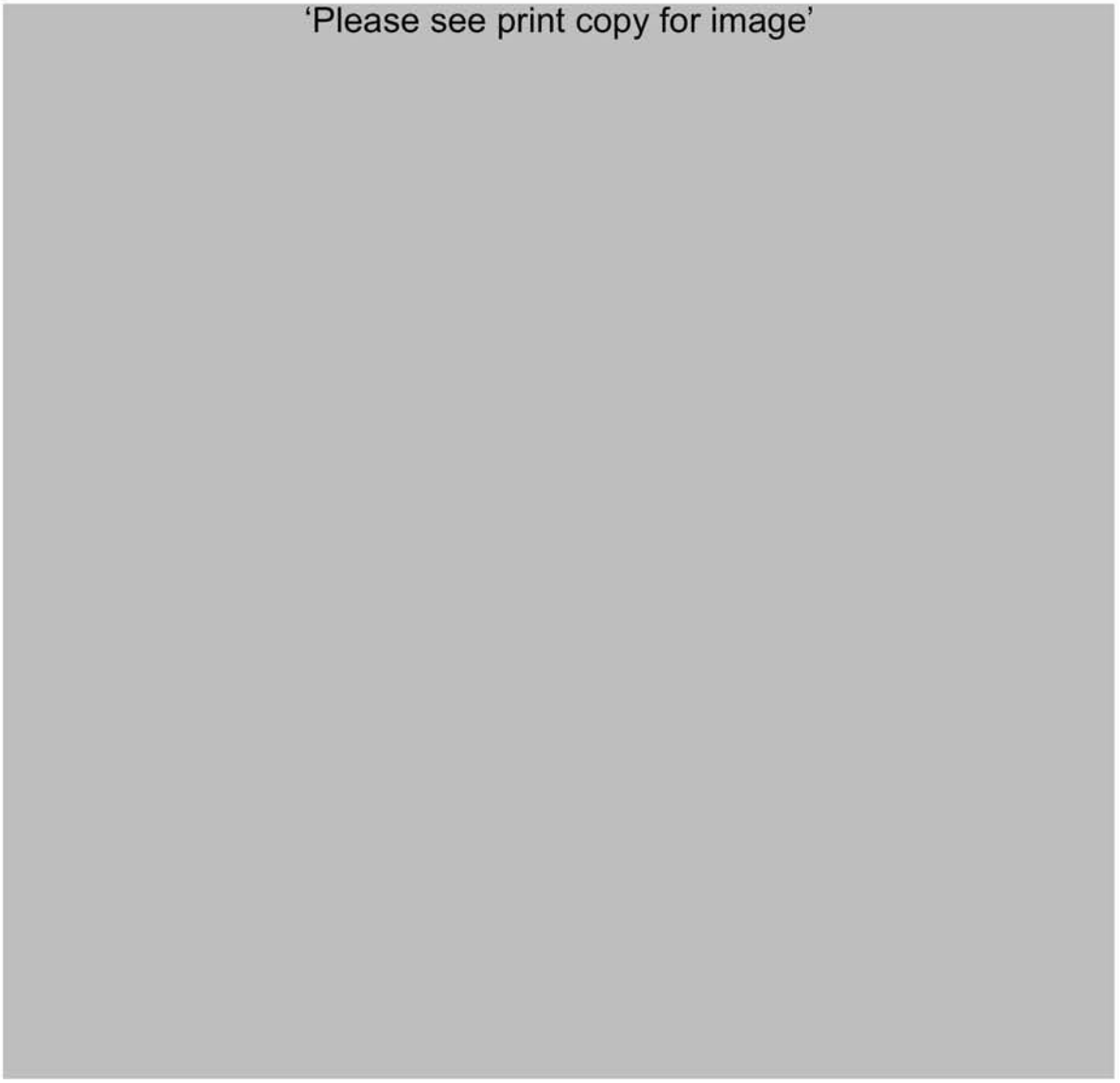

Figure 3.4. Flower colours for *Hardenbergia violacea* (*A*), *Diuris maculata* (*B*) and *Daviesia ulicifolia* ssp. *ulicifolia* (*C*) plotted in a colour hexagon.The purple flowers of *H. violacea* are well separated from *D. maculata* in the bee vision system, being about 0.22 colour hexagon units apart and would be distinguishable by a bumble bee almost 100% of the time (Dyer and Chittka 2004). The flowers of *D. maculata* are only separated by about 0.058 colour hexagon units from *D. ulicifolia* ssp. *ulicfolia* and could be expected to make colour-based foraging errors about 25% of the time.

Pollen Analysis

At the start of flowering of *D. maculata* few other flowers were evident apart from the epacrid shrub *Lissanthe trigosa* ssp. *subulata* and occasional *H. violacea*. Male *T. venustus* bees were seen to visit frequently the epacrid, but as legume flowers became available they were clearly preferred. By the time of peak flowering of the orchid, *H. violacea* flowers were abundant as well as some early *D. ulicifolia* ssp. *ulicifolia*. Most of the male and female *T. venustus* bees captured were found to have only legume pollen of both *D. ulicifolia* ssp. *ulicifolia* and *H. violacea*. Figure 3.2*c* shows a photomicrograph of pollen washed of a female *T. venustus* bee. The larger, triangular grains, about 35 μm across are from *H. violaea*. The smaller, almost spherical grains, about 19 μm across are from *D. ulicifolia* ssp. *ulicifolia*.

Nectar sampling

On 24 August 2001, inflorescences were covered with nylon gauze bags overnight and individual flowers sampled for nectar at 1400 hours the next day. Three of eight sampled flowers of *D. ulicifolia* ssp. *ulicifolia* had measurable nectar (2, 8 and 1%, mean = 3.7%) compared to 9 of 10 flowers of *H. violacea* (mean of 10 flowers = 4.3%). None of ten flowers of *D. maculata* sampled had detectable nectar. These data would indicate that both legumes offer a nectar reward and support the assertion of Beardsell *et al*. (1986) that *D. maculata s.l.* does not offer a nectar reward.

Discussion

Orchids suspected of being food source Batesian mimics are expected to show a number of characteristic features. Kjellsson *et al*. (1985) list these features, which are quoted

here: "The following conditions should indicate an operating floral mimicry system, where the model is a flowering plant species which is normally visited by a pollinator in search of food: (1) visual and morphological similarity (including colour, shape and scent) between the flowers of the model and the mimicking species; (2) lack of any kind of food reward in the mimic; (3) both the model and the mimic are visited by the same pollinator; (4) the mimic has higher fitness (seed production) when taking part in an operating mimicry system than when it grows alone; (5) the population of mimics should be relatively small in number of flowers compared with the model species population; (6) the model and the mimic should not be too widely dispersed either in space or in time of flowering."

At Scheyville National Park flowers of two legume species of contrasting colour (purple vs. yellow to human eyes and also very distinct in the bee vision system) are visited by the legume specialist bee *T. venustus*. *Trichocolletes* species are fast-flying medium-sized, solitary native bees, many of which are legume specialists (Cardale 1993). Males of *T. venustus* appear before females. This is a common situation among solitary bees (O'Toole and Raw 1991). Solitary bee females, in addition to supplying their own energy needs, must stock a nest with the food required to allow development of eggs through to the adult stage. Males, however, need only forage to supply their own energy needs, and much of their activity appears connected with finding mates. Males of *T. venustus* do not appear to be territorial, but fly in a general search pattern over a patch where they frequent plants in flower likely to harbour females (JI, personal observation). On finding a female, a male will make a rather clumsy and boisterous sexual advance which is mostly unceremoniously rejected, since the females are monandrous and the majority seen will have already been mated. *D. maculata* at

Scheyville National Park are often pollinated by male bees early in the orchid flowering season when flowers of *D. ulicifolia* ssp. *ulicifolia* (the putative model) are uncommon, suggesting that these naïve bees probably visit any flowers which resemble those of legumes, most commonly yellow in colour, but also frequently purple. The later appearance of large numbers of female *T. venustus*, coinciding with the main flowering of *D. ulicifolia* ssp. *ulicifolia* would, however, not seem to preclude pollination by females, which may occur, but clearly less commonly than by males. The pollination mode can be best described as guild mimicry of legume flowers (Dafni and Bernhardt 1990) and our data therefore provides support for the findings of Beardsell *et al.* (1986). Our data meet criteria 2, 3 and 5 above. Criterion 4 is more difficult as *D. maculata* was found to be strongly associated with legumes at all sites where we found it. This does not preclude the possibility of this orchid occurring in the absence of legumes at some sites within its range, but such occurrences would be unusual. As male *T. venustus* bees were noted to be visiting *Hardenbergia violacea* more than *Daviesia ulicifolia* spp. *ulicifolia*, it it is unclear whether the putative mimic *Duiris maculata* shows colour similar to a model pea species, and therefore cannot be shown to meet criterion 1.

Beardsell *et al.* (1986) showed that at their study site *D. maculata s.l.* and sympatric legumes are closely matched in flowering time and satisfy well criterion 6 above. However, at Scheyville National Park, the orchid was found to have a peak flowering some 2 weeks earlier than the most obvious putative model species, *D. ulicifolia* ssp. *ulicifolia*. This feature, which in this case appears to exploit naïve male *T. venustus* bees, is apparently quite common in *Diuris* (Dafni and Bernhardt 1990) and is in contrast to the findings of Beardsell *et al.* (1986). Beardsell *et al.* (1986) asserted that *D. maculata s.l.* lacked nectar, but they did not test this, whereas we employed a sensitive

assay to confirm that this orchid does indeed lack nectar. This is important because, contrary to common assertions in the literature, a number of *Diuris* species do produce nectar (e.g. *D. sulphurea*: see Chapter 6, *Diuris alba*: see Chapter 4) and this needs to be checked for all species. The finding of the initial appearance of orchid flowers *before* the appearance of model flowers (with, however significant overlap in flowering phenology) is somewhat at odds with classical expectations of Batesian mimicry. Bee floral visitors can be expected to some extent to be pre-adapted to visit flowers likely to yield a reward. Mimics have been shown to benefit from the magnet effect of abundant model plants (Peter and Johnson 2008). In the relative absence of model plants, a mimic may do well if it appears just before the main flowering of the model if pollinators are present at the time.

Elliott and Ladd (2002) studied the pollen limitation of fruit set in Western Australian terrestrial orchids, including *Diuris brumalis*, a putative legume mimic. When one flower each in 40 plants was hand-outcrossed, ~90-100% of hand-pollinated inflorescences formed fruit. That contrasted with low natural pollination of ~10-15% of flowers (as inferred from their data histogram) in degraded woodland where legumes were absent. *D. brumalis*, a non-rewarding species, showed marked pollen limitation, with much higher fruit set found in hand-pollinated flowers than open-pollinated flowers. However, fruit set for open-pollinated flowers was similar to that found by Beardsell *et al.* (1986) and the present study. Neither the results presented in Beardsell *et al.* (1986) nor the data in the present study suggest abnormal reproductive success for *D. maculata s.l.*, since both studies report the presence of abundant putative model plants and putative pollinators. Although it is likely that habitat fragmentation and reduction in population size result in pollination suppression, low reproduction rates

nevertheless appear common. Thus, the challenge for pollination studies, with implications for conservation strategies, is to distinguish between low (normal) and dangerously low (compromised) pollination rates, not a trivial matter in a country so dramatically altered by European settlement that undisturbed habitats may be rare, or non-existent for some orchid species. The very large numbers of tiny seeds that result when fruit are formed may be sufficient to maintain stable populations in orchid species with low reproductive success, whether natural, or artificially suppressed.

Batesian mimicry (and the related guild mimicry) poses a puzzle in that it is to be expected that flowers offering a nectar reward would attract more visitors with greater floral constancy than those offering no reward. However, the limited available comparative data suggests that this is not necessarily the case (Johnson 1994). Batesian mimics of a specific model species, tend to be rare. Guild mimicry by an orchid may be distinguished from Batesian mimicry, since guild mimics may have several, or even multiple model species, but otherwise the mechanics of pollination may be indistinguishable. However, the flexibility of pollination of a guild mimic where the (presumably adequate) low reproductive success can potentially be achieved at many different sites, with different model and pollinator species, would appear to account for the ability of *Diuris maculata* to become a common and widespread orchid. Within a 200-km radius of Sydney, a number of taxa can be found that are closely related to *D. maculata* (e.g. *D. semilunulata*, *D. aequalis*). Both of these species also appear to be mimics of pea flowers and show remarkable similarity in colour and form to sympatric pea flowers (JI, unpubl. obs.). Another group of taxa, related to *D. punctata*, although pea-like in floral form, mostly have flowers of pinkish-purple coloration. Preliminary work shows that these putative pea mimicking species are mainly found in the presence

of yellow flowering peas, and that being of the 'wrong' colour does not appear to result in lower visitation by bees foraging on these pea flowers (see Chapter 6 for further details). Great floral variability within and between *Diuris* populations causes considerable taxonomic problems. However, comparative photographs showing the range of variation between pea flowers and sympatric *Diuris* orchid flowers show a comparable high degree of variation in size, coloration and markings of both orchids and peas. This suggests that high variability is characteristic of these bee-pollinated flowers. Further work needs to be done to establish why some *Diuris* species seem to show selection for close mimicry of pea flowers, while other species achieve a similar (or higher) reproductive outcome by apparently employing much looser mimicry.

There is a considerable literature on visual cues of bee-pollinated flowers (Barth 1991; Proctor *et al.* 1996). Mellitophilous flowers tend to be zygomorphic, with complex three-dimensional structure. They tend to have bright and contrasting colours with visual and also often scent-based nectar guides. Bee colour vision is also known to be well developed and to extend into the UV range. A diverse array of angiosperm lineages possess flowers with an UV colour component perceptible to hymenopteran floral visitors (Chittka *et al.* 1994; Heuschen *et al.* 2005) and these often have UV-absorbing nectar guides (Jones and Buchmann 1974).

Australian bee-pollinated legumes are known to frequently possess UV colours and nectar guides (Kay 1987), so it is only reasonable to expect to find comparable UV patterns in *Diuris* spp. which are putative legume mimics. These have been found *D. maculata* and its sympatric legumes at Scheyville National Park and provide additional support for mimicry in this orchid. Our colorimetric data show that *D. maculata* and the

sympatric pea flower *Daviesia ulicifolia* ssp. *ulicifolia* are sufficiently close in colour for bees foraging on the pea flowers to make colour-based foraging errors and to visit the orchid by mistake. The flowers of most species of *Diuris* are similar to sympatric pea flowers in floral form, but many are dissimilar to sympatric peas in colour. It seems that in some *Diuris* taxa selection for for colour mimicry may be strong, but that in other cases it appears to be weak. Available data for *D. maculata* are consistent with strong colour mimicry of *D. ulicifolia* ssp. *ulicifolia*, but not *H. violacea*. However, yellow-flowered peas are more numerous at the many sites where *D. maculata* occurs, and this orchid, or an ancestor, may have evolved colour similarity to the colour of these peas.

Some further details of the mechanics of the bee's visitation of pea flowers, incorporating knowledge of the bee's sensory perception (Barth 1991), can help us develop a model of the way the same bees visit *Diuris* flowers. This model fits our observations based on examination of many close-up photographs of various bee species foraging on a range of pea species. Figure 3.2*b* shows a female *T. venustus* bee visiting a flower of *H. violacea*. The bee, having been attracted by the bright purple colouring (including an UV component), complex three-dimensional form and contour outline of the flower, proceeds to land on the flower. This flower, in common with many mellitophilous flowers, shows enrichment of longer wavelengths towards the centre of the flower (i.e. outer purple colour is composed of UV + blue + yellow wavelengths and the greenish nectar guide spots at the base of the standard petal lack blue and UV components - see Fig. 3.3*c*, *d*), as well as a fragrance gradient which a bee can orient in three-dimensional space. Together these direct the bee to the centre of the flower where the UV-absorbing nectar guide acts as a cue for the bee to extend its

tongue in search of nectar. The bee is then positioned to access the concealed pollen within the keel. This description emphasises the importance of the nectar guide in foraging behaviour.

The brief visits of bees to *D. maculata* flowers have not been photographed, so the events of visitation must be inferred based on known details of visits of bees to pea flowers. When visiting a *Diuris* orchid flower, a bee would perceive similar long range visual cues to a pea flower and, upon landing orient to the false nectar guide, which mainly comprises callous ridges of the labellum (Fig. 3.3*a*, *b*) (*D. maculata* does not appear fragrant, but may have a fragrance perceptable to bees). We expect that the bee would extend its tongue to seek the absent nectary and, on failing to find it, fly to another flower. On four occasions, we observed males of *T. venustus* fly from a flower of *H. violacea* to a nearby flowering plant of *D. maculata* and then to one or more additional *Diuris* plants in quick succession, after which they quickly flew away. This model, which emphasizes the role visual cues and nectar guides in the bee's foraging behaviour differs in some respects from that suggested by Beardsell et al. (1986) and reviewed by Bower, as cited in Pridgeon *et al.* (2001). In particular, this model of orchid visitation provides an explanation of how the bee precisely orients to the orchid flower such that the viscidium of the pollinarium attaches to the centre of the face of the bee.

We have found that orchid pollinaria are found precisely positioned on the faces of captured bees, but accumulating observations suggests that the probability of a pollinarium being collected on a visit to a flower of *D. maculata* is much less than 100%. It is also possible that bees may learn from experience to detect the mimic (possibly after alighting on the flower). However, if visits are infrequent, learning

opportunities also would be limited. If floral visits by bees involve pressing of the head against the standard petal of the pea flower, as suggested by Beardsell *et al.* (1986), then similar visits to orchid flowers would be expected to result in pollinaria collection in more or less 100% of orchid flower visits. By contrast, we expect female function (transfer of collected pollen to the stigma of another flower) to be more efficient than male function. The large size and prominently forward-projecting position of the pollinarium on the front of a bee's head and the large size of the stigma relative to the viscidium should result in a collected pollinarium contacting the stigma with higher probability than the bee's head would contact the viscidium in the first, male-function visit.

Since the finding of UV patterns in the genus *Diuris* have not been published before, it is appropriate to provide further examples. Figure 3.5 shows *D. maculata* from Stringy Bark Ridge, Pennant Hills, with sympatric flowering species. Figure 3.5 shows visible light photographs in (*a*) colour and (*b*) black and white respectively of two *D. maculata* flowers at the bottom, with the yellow-flowered sympatric legumes *Bossiaea obcordata* at centre left and *Dillwynia retorta* at the top. On the centre-right, the white-flowered and probably mostly fly-pollinated daisy bush *Olearia* sp. is shown as an 'outgroup'. Note that in Fig. 3.5*c* the comparative UV photograph shows that the two legume species and the orchid reflect similarly in the UV and UV-absorbing true and false nectar guides respectively. As is common with white flowers, the *Olearia* sp. does not produce UV reflectance (Kevan *et al.* 2001) and appears dark in this illustration. *D. maculata* can be considered to have rather generic pea-flower-mimicry characteristics. This is in contrast to *Diuris aequalis*, which occurs in Kanangra-Boyd National Park. This rare species, also a putative pea mimic, shows a remarkable similarity in shape and

colour to the sympatric legume *Gompholobium huegelii,* which is shown in comparative photographs in Figure 3.6. These flowers are plain yellow under visible light and are shown in (*a*) colour and (*b*) black and white respectively. In Figure 3.6*c* both species can be seen to have overall UV reflectance, with prominent UV-absorbing nectar guides. Similar UV patterns have been observed in other *Diuris* species, including *D. sulphurea* (various sites in NSW), *D. punctata* (Tallong, NSW), *D. chryseopsis* (Ilford, NSW), *D. conspicillata* (Esperance, WA) and numerous others (J.O. Indsto, unpubl. data), from which it can be concluded that such patterns are the norm for this genus, with few exceptions.

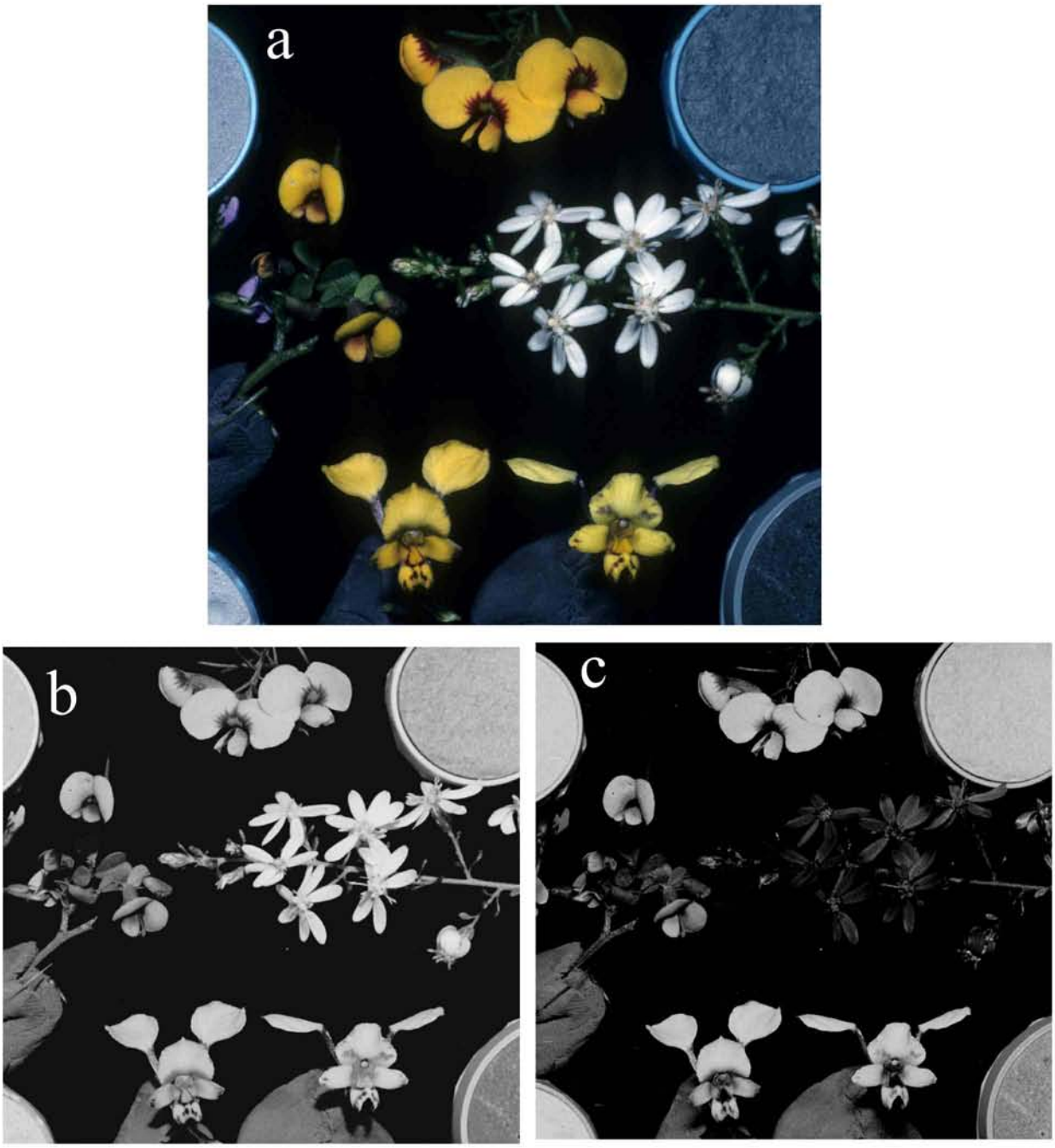

Figure 3.5. Comparative human visible range (HVR) and UV images of orchid and sympatric legume flowers. (*a*) HVR colour and (*b*) black and white images respectively, of *Diuris maculata* from Stringy Bark Ridge, Pennant Hills at bottom centre (orchid flowers are about 20 mm across), with *Bossiaea obcordata* at left and *Dillwynia retorta* at top, and the white daisy *Olearia* sp. at centre right. (*c*) Comparative UV images. Note that the orchid and legume flowers reflect have overall UV reflectance and have false and true UV-absorbing nectar guides respectively. The *Olearia* sp., like many white flowers does not reflect under ultraviolet light.

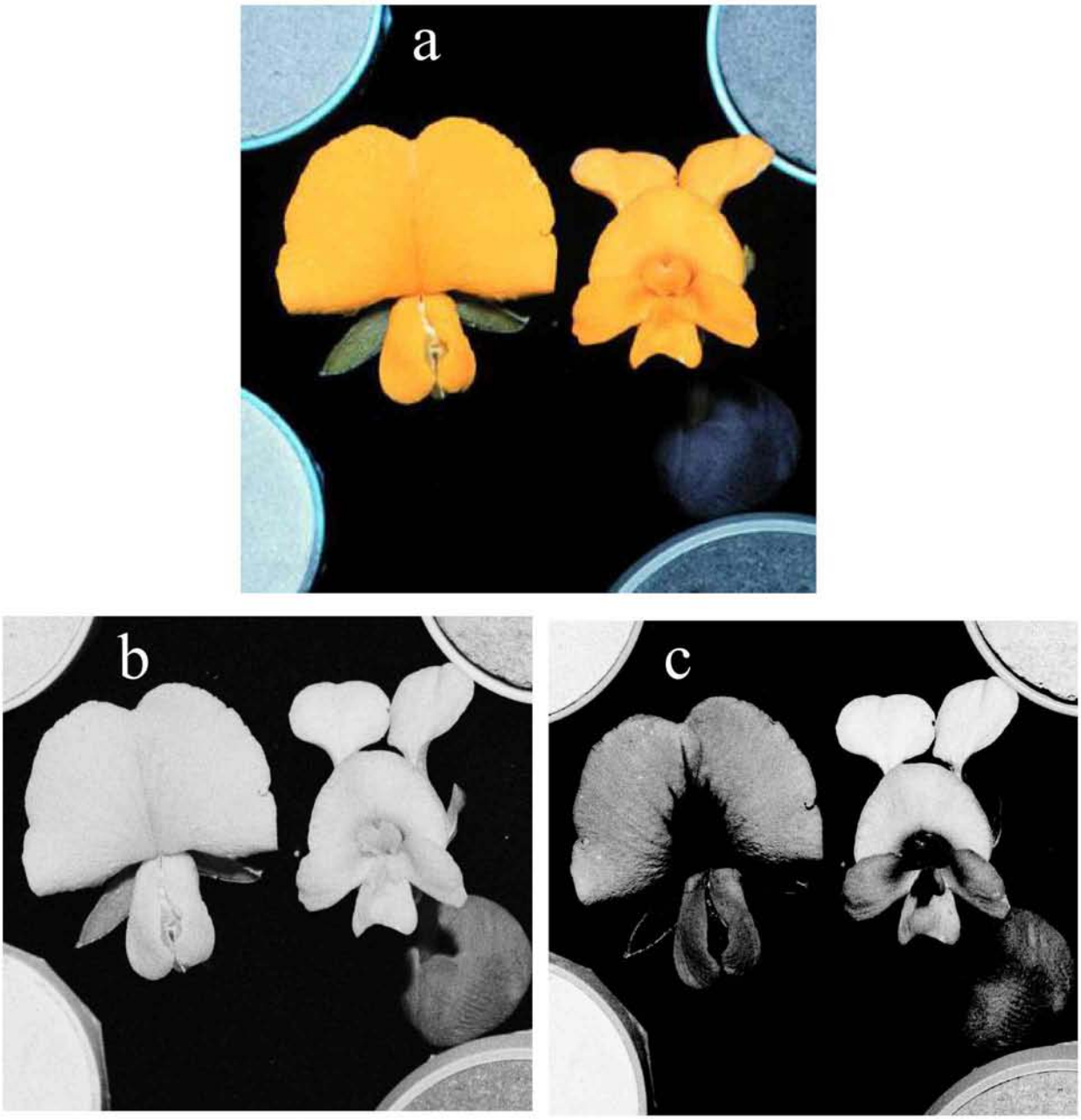

Figure 3.6. Comparative human visible range (HVR) and UV images of orchid and sympatric legume flowers. (*a*) HVR colour and (*b*) black and white images respectively, for the legume *Gompholobium huegelii* at left and the orchid *Diuris aequalis* at right. (*c*) Comparative UV image. Both the legume and orchid show remarkably similar UV-absorbing false and true nectar guides, respectively. Note: The standard petal of the *G. huegelii* flower is about 25 mm across.

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Chapter 4

Generalised pollination of *Diuris alba* (Orchidaceae) by small bees and wasps

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Prologue

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(Orchidaceae) and wasps. *Australian Journal of Botany*, 55: 628-634. Boris Branwhite and John Riley provided advice on orchid colony location and timing of flowering. Peter Weston assisted in field work and in the identification of voucher specimens. Adrian Dyer carried out reflectance spectrophotometry and colorimetric analysis. Michael Batley also contributed to field work and identified all bees collected.

Abstract

Most *Diuris* species possess flowers of pea-like form and colour, and occur in association with flowering peas of the tribe Mirbeliae. Previous studies of the pollination of *Diuris maculata sensu lato* have found evidence for guild mimicry of pea flowers. The flowers of *Diuris alba* are also pea-like in form but not in colour, and this species is frequently found in habitats where peas are uncommon or absent. We investigated the pollination of *Diuris alba*, which we expected may have a distinct pollination system, at Lake Munmorah, New South Wales. Many *Diuris* species lack floral rewards, but *D. alba* produced a small amount of nectar. Flower visitors, and hence putative pollinators, were hymenopteran: mainly female *Exoneura* (Apidae) bees, but also the wasps *Eurys pulcher* (Pergidae) and a *Paralastor* species (Eumeninae). Reproductive success of *D. alba*, both in woodland containing abundant *Dillwynia retorta* and in heathland where this pea was absent, was higher than in the previously studied *D. maculata s.l.* We suggest that the pollination of *D. alba* is more generalised than that found in the legume guild mimic *D. maculata s.l.* Although its flowers may display structural similarity to pea flowers, other characteristics suggest that its pollination system has diverged from a presumed pea-mimicry ancestral condition.

Introduction

Many *Diuris* species are thought to be mimics of pea flowers of the tribe Mirbeliae. However, apart from the studies of Beardsell *et al.* (1986) and Indsto *et al.* (2006, in press), references to *Diuris* pollination in the scientific literature are largely anecdotal (see Bower, 2001 and van der Cingel, 2001 for reviews). The only reference we have found for *Diuris alba* pollination is by Jones (1988) who refers to simple pollination of *Diuris punctata* and *D. alba* by small bees. Beardsell *et al.* (1986) studied *Diuris maculata sensu lato* at a site near Melbourne and suggested this orchid is a mimic of peas of the genera *Daviesia* and *Pultenaea*. This form of mimicry, of florally similar sympatric pea species that may be regarded as Mullerian mimics, has been termed guild mimicry to indicate a more general form of Batesian mimicry (Dafni and Bernhardt 1990). The pea-like flowers of *D. maculata* possess comparable UV nectar guides to those found in the flowers of pea genera including *Pultenaea*, *Daviesia*, *Dillwynia* (Fabaceae: Mirbelieae) and *Hardenbergia* (Fabaceae: Phaseoleae) (Kay 1987; Indsto *et al.* 2006); although, as *D. maculata* lacks nectar, these are false nectar guides. Indsto *et al.* (2006) pointed out that these provide additional supporting evidence for pea mimicry in *Diuris* species and that these are present in most *Diuris* taxa.

To our knowledge the only current study on the pollination of *Diuris alba* is by Jones (1988), who referred to 'simple pollination' of *Diuris punctata* and *D. alba* by small bees. Several features of *Diuris alba* suggest that it may not depend on guild mimicry of peas for is pollination. Firstly, substantial populations, often of hundreds of plants, are found at sites where peas are uncommon, or absent. Second, although the flowers of *D. alba* are pea-like in form, their colour, as the name implies, is basically

white with variable purplish suffusions of the labellum and sometimes other flower parts (see Figure 4.1). The callous ridges at the base of the labellum are yellow, contrasting clearly with the rest of the flower. On the basis of colour, *D. alba* flowers do not appear to mimic pea flowers, particularly the yellow "egg and bacon" types in the genera *Pultenaea*, *Daviesia* and *Dillwynia*. Floral features of this orchid are strongly suggestive of bee pollination (Barth 1991). Many species of *Diuris* are pea-like in form and colour, suggesting pea mimicry may not only be widespread in the genus, but probably ancestral. This hypothesis is supported by a DNA-based cladistic analysis of *Diuris* (Indsto *et al.* 2009), in which *D. sulphurea*, which has pea-like flowers, forms a sister group to the rest of *Diuris*. Our investigations of pollination of *Diuris alba*, which is expected to show differences to other *Diuris* species, may be better understood with the benefit of phylogenetic considerations.

Many *Diuris* species are thought to be pea-flower mimics, since most show a general resemblance in form and often of colour to pea-flower species, with which they are commonly sympatric. This suggests that pea-flower mimicry is most likely the ancestral pollination mode in the genus. The pollination of only one species, *Diuris maculata s.l.* has been studied in detail (Beardsell *et al.* 1986; Indsto *et al.* 2006) and has been proposed to be a pea guild mimic. *Diuris alba* did not appear to fit this syndrome, so we gathered data for this species and contrasted these data with comparative data for the putative guild mimic *Diuris aurea*.

Figure 4.1. *Diuris alba* from Lake Munmorah, New South Wales. Flowers are about 25 mm across.

Materials and Methods

In the present study we gathered basic data on the pollination mechanism of *Diuris alba* by methods similar to those used previously by Beardsell *et al.* (1986), but with additional data from nectar testing, AFLP fingerprinting of orchid pollinaria to confirm putative pollinators, UV-reflectance photography and colorimetric analysis of flowers (Indsto *et al.* 2006).

To explore the possibility that the pollination mechanism of *D. alba* may be distinguished from pea flower guild mimicry, we chose two contrasting study sites. At Site A, scattered plants *D. alba* occurred in the presence of the abundant and locally dominant pea shrub *Dillwynia retorta*. A second study site, Site B, was in heathy habitat where pea flowers were not dominant. This is frequently the case for locations in which *D. alba* occurs at Lake Munmorah. As *Diuris aurea* also occurred at these study sites, and this species has floral characteristics that suggest it is a pea guild mimic similar to *Diuris maculata*, we therefore collected pollination data for this species. We felt that if *D. alba* was not a pea mimic, then the way its pollination system responded to the differences between sites should differ form the putative pea mimic *D. aurea*.

The comparative reproductive success of *Diuris alba* was studied at two ecologically contrasting sites in order to compare its reproductive success against expectations for a legume (pea flower) guild mimic. Pollination data for the sometimes sympatric *D. aurea* were also included where these were informative about the pollination of *D. alba*.

Site A was at Lake Macquarie State Recreation Area, Chain Valley Bay, on the eastern side of Chain Valley Bay Road, ~2.5 km from the Pacific Highway and opposite Houston Avenue. This site consisted of woodland with a grassy understorey and extensive patches of the legume shrub *Dillwynia retorta*. Other less abundant flowering shrubs included *Pimelea linifolia* ssp. *linifolia*, *Mirbelia rubiifolia*, *Grevillea sericea*, *Lambertia formosa* and *Comesperma ericinum* as well as the herb *Burchardia umbellata*. In 2001, ~80 flowering plants of *D. alba* and 20 *D. aurea* were present in an area of 20 m by 6 m. Smaller numbers of scattered *Diuris* and the terrestrial orchids *Thelymitra ixioides* and *Calochilus paludosus* were also present nearby.

Although occasional flowering plants of *Diuris alba* may be seen in late August, the main flowering generally begins early September, reached a peak about mid September and was largely finished by the end of the month. *D. aurea*, which is occasionally sympatric, follows a similar flowering pattern, but delayed by ~10 days. The main flowering period of *D. retorta* extends from mid-August to mid-September with flowering largely in decline by the end of September. The other flowering shrubs and *Burchardia umbellata* flower in a similar pattern.

Site B was at Munmorah State Recreation Area near Lake Munmorah. A *D. alba* population, with sympatric *D. aurea* occurred near the western side of Blue Wren Drive, 800 m from the Pacific Highway. The site is about 1 ha in total area and consists of grassy heath adjacent to woodland, with no *Dillwynia retorta* present. Overall, the

site was characterised by a diversity of flowering plants with *Pimelea linifolia* ssp. *linifolia* being the most abundant. The shrubs listed for the Lake Macquarie State Recreation Area site, other than *D. retorta,* were all present, as well as *Philotheca salsolifolia* and *Isopogon anemonifolius*. The peas *Hardenbergia violacea*, *Pultenaea rosmarinifolia* and *Bossiaea ensata* were common, but not dominant.

Observations of putative pollinators, pollen analysis and pollination statistics

Insects visiting flowers were caught by netting, asphyxiated over dry ice (this method has the advantage of reducing the likelihood of pollen cross transfer between captured bees that can be a problem when bees are asphyxiated in a killing jar), and stored at -20°C until required for pollen removal and identification. Representative samples have been lodged with the Australian Museum. Pollen was washed from the bodies of all captured insects with 70% ethanol onto uncharged microscope slides and, upon drying, stained with Calberla's fluid (Ogden et al. 1974). Reference samples of pollen were taken from voucher specimens of sympatric flowering plants and Diuris species. Beardsell et al. (1986) included observations only of insects carrying orchid pollinaria, whereas in the present study representatives of all hymenopteran flower visitors of both peas and orchids were captured and recorded, in order to give an overview of the spectrum of floral visitors.

Nectar sampling

For detecting small amounts of sugars, a method similar to that of Gross was used (Gross 1990). Nectar testing was carried out on *Dillwynia retorta*, *Diuris alba* and *D. aurea* on 19/9/03 at 1130 hours on plants at the Lake Macquarie State Recreation Area,

Chain Valley Bay site. The calibration of the refractometer was checked with distilled water between samples. Fresh looking flowers, which were not covered overnight, were sampled as a representative of the floral reward likely to be encountered by pollinators. False negative results might be obtained if flowers sampled had been recently visited, but this was considered unlikely for all of a number of randomly sampled flowers.

DNA analysis of pollinaria and remnants using AFLP

The species origin of orchid pollinaria removed from captured bees was determined using a modified AFLP protocol (Indsto *et al.* 2005, 2006). The phylogenetic relationships of *Diuris* species have been studied by AFLP (Indsto *et al.* 2009), in part on the basis of sampling of the species in the present study. We found a clade of species related to *Diuris punctata* (and including *D. alba* and *D. aurea* in the present study), which is distinct from a clade of species related to *Diuris maculata* (and containing *D. praecox*). Species within these very distinct clades show little genetic heterogeneity. However, *D. alba* and *D. aurea* can be distinguished from each other by AFLP on the basis of just one band. Pollinaria removed from captured insects generally yielded abundant DNA for testing. *D. praecox* was also present nearby, but has a main flowering period about a month earlier and was only sporadically still found in flower during the flowering period of *D. alba*. Other orchid species present at the study sites, or nearby included the diurid species *Thelymitra ixioides* and *Calochilus paludosus.* These were also expected to have pollinaria morphologically similar to *Diuris* spp., that might be confused with those of *D. alba*.

UV-reflectance photography

Flowers of *Diuris alba, D. aurea* and putative model flowers were photographed with and without UV-transmitting filters as described elsewhere (Williams and Williams 1993; Indsto and Weston 1999) using patches which reflect evenly throughout the visible and near-UV range (Dyer 1996). These patches are helpful in avoiding spurious (i.e. uncalibrated) comparisons of visible and UV-range images.

Colorimetric analysis

To quantify how pollinating insects are likely to perceive flower colours it is possible to measure the spectral reflectance properties of flowers and plot the loci in a colour space. We used a hexagon colour model (Chittka 1992) which is generally applicable for hymenopteran insects with trichromatic vision (Backhaus 1991). Reflectance properties of fresh flowers were measured with a double beam Varian DMS100 spectrophotometer relative to a 100% standard, and loci in colour space were calculated considering the spectral sensitivities of honeybee colour receptors (Menzel and Backhaus 1991; Chittka 1992) as described in detail in Indsto *et al.* (2006). This modelling of colour perception allows a meaningful interpretation of how similar colours might be perceived (Dyer and Chittka 2004). In general, that study showed that colour distances less than ~0.05 hexagon units are poorly discriminated by bees (i.e. bees are likely to generalise on these similar colours), whereas colours separated by 0.15 or greater units are well discriminated by bees. Colours between these values are discriminated, but with some errors in judgement.

Results

Observations of putative pollinators

By far the most abundant flower visitor at Site A was the introduced European honeybee, *Apis mellifera*, which outnumbered all other bees by at least 10:1. Other commonly seen insects that were commonly seen visiting flowers included small butterflies and syrphid flies, both of which visited *Dillwynia retorta* and *Diuris aurea,* but which did not appear to be involved in orchid pollination.

The presence of orchid pollinaria on foraging honeybees was easily observable and it is estimated that far fewer than 1% of these carried orchid pollinaria. One honeybee captured with orchid pollinaria was found by AFLP fingerprinting to be carrying those of *D. aurea* (Fig. 4.2*b*, also see Table 4.1). Bees of *Exoneura* spp. (Hymenoptera: Apidae) were frequently seen visiting *Dillwynia retorta* (see Figure 4.2*a*) and *Diuris aurea*, and these were the only bees seen to visit *D. aurea*. No visits of bees, or other insects, were observed for *D. alba* at Site A. Visits to *D. aurea* by bees of *Exoneura* spp. were frequent, but none of the bees was seen to collect pollinaria of this orchid. One *Exoneura* sp. captured on *Dillwynia retorta* carried what appeared to be the remnants of orchid pollinaria, but gave a failed result with AFLP. In the years 2001 and 2002, pollinator observations totalled ~8 h and 4 h, respectively.

Observations of putative pollinators at Site B were made during 2002 and 2003 (see Table 4.2). No insects were seen to visit either of the *Diuris* species in 2002 at this site, although one honeybee was seen to approach *D. alba*, but not land. In 2003, however, numerous visitors to *D. alba*, but not *D. aurea*, were seen. We did not collect data for insect visitors to plants other than orchids at this site.

Ten insects, all hymenopteran, and mostly *Exoneura* spp., were seen visiting *Diuris alba* at site B in 2003. The bees of *Exoneura* spp. are small (~8 mm in length), slender and wasp-like in appearance. The smooth bodies of these bees collected very little pollen, so pollen counts were very low. Two out of eight of these small bees carried *D. alba* pollinaria, as confirmed using AFLP analysis. One of these, carrying two pollinaria of *D. alba*, is shown in Fig. 4.2*c*. At this site these bees visited a range of flowers, on the basis of evidence from pollen washes, including *Pultenaea rosmarinifolia*, *Comesperma ericinum* and another legume, most likely *Hardenbergia violacea.* A wasp, *Eurys pulcher* (Hymenoptera: Pergidae) was captured on *D. alba* carrying *Diuris* orchid pollinaria, but the AFLP analysis profile obtained was not well enough resolved to distinguish between *D. alba* and *D. aurea*. These wasps are common visitors to a range of flowers. A small parasitic wasp, *Paralastor* sp. (Hymenoptera: Eumenidae), was also captured on *D. alba*, but was not carrying orchid pollinaria. All these insects captured on *D. alba* are of similar size and shape. The putative pollinators of *D. alba* were small, slender insects that approached the orchid with a slow, bobbing flight and tended to remain for at least 10 s on a particular flower. The insects were easily caught by lowering a net over the orchid inflorescence. The behaviour of these insects contrasts with that seen for medium-sized fast-flying bees seen visiting the putative guild mimic, *Diuris maculata*, at Scheyville National Park (Indsto *et al.* 2006). Visits to *D. maculata* were infrequent and very brief and all bees caught with orchid pollinaria were from peas adjacent to the orchids.

No insects were found to be carrying pollinaria of *Diuris aurea* at site B in 2003.

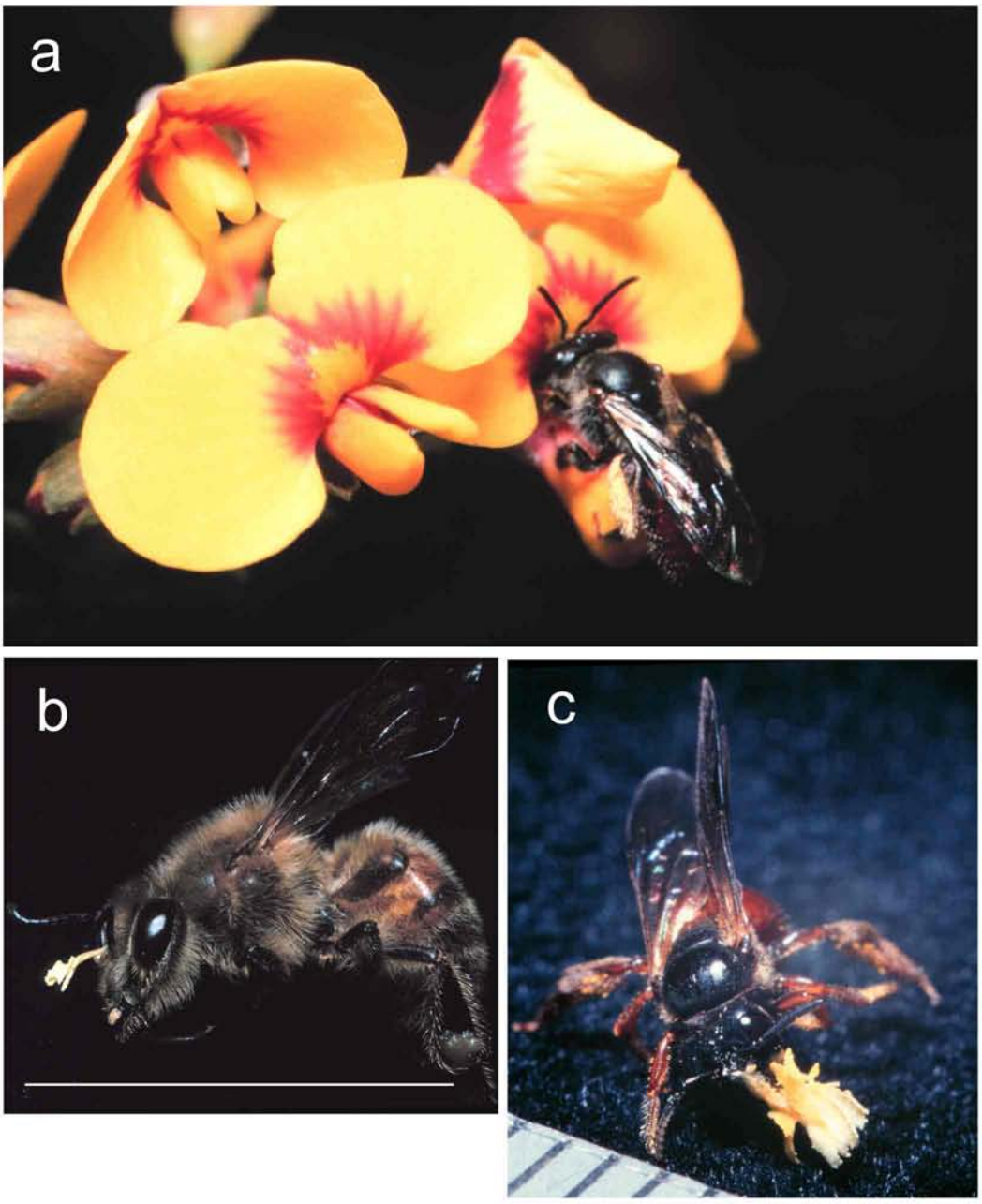

Figure 4.2. Images of bees. (a) *Exoneura* sp. bee foraging on *Dillwynia retorta*. Note the precise orientation of the face of the bee to the UV-absorbing patch at the base of the standard petal. Bees captured with orchid pollinaria: (b) Honeybee captured on *Dillwynia retorta* carrying *Diuris aurea* pollinarium (see Table 4.1), scale bar = 13 mm (c*) Exoneura* sp. bee carrying two pollinaria of *D. alba* (see Table 4.2). Note that both of these bees carry the pollinaria on the lower half of the face. Orchid species source of pollinaria were confirmed by AFLP. Note: these gracile bees are about 6 mm in length.

Table 4.1. Captured bees from 2001, 2002 and 2003 orchid flowering seasons at Site A. Bees with orchid pollinaria are shown in bold type.

Bee species	**Sex**	**No. Caught**	**Host Plant**	**Pollen Analysis Summary**	**AFLP Analysis**
Leioproctus rectangulatus	all F	4	all on *Dillwynia retorta*	only *Dillwynia* pollen seen	
Apis mellifera	F	2	on *Pimelea*	both with few *Pimelea* grains	
Apis mellifera	F	1	On *Dillwynia retorta*	*Dillwynia* only	
***Apis mellifera* – with orchid pollinarium**	***F***	1	on *Dillwynia retorta*	*Dillwynia* plus some orchid pollen	pollinarium matches *D. aurea*
Exoneura sp. – with pollinarium remnants	F	1	on Dillwynia retorta	mostly *Dillwynia* pollen plus some unidentified	null result for pollinarium remnants
Exoneura sp.	F	1	on *Pimelea*	mix of *Pimelea* and *Dillwynia*	
Exoneura sp.	F	1	on *Diuris*	*Dillwynia* only	

			aurea		
Exoneura sp.	All F	5	on *Dillwynia retorta*	mostly *Dillwynia*, plus a few unidentified	
Lasioglossum orbatum	F	1	on *Dillwynia retorta*	few *Dillwynia* grains	
Megachile leucopyga	F	1	on *Dillwynia retorta*	*Dillwynia* only	
Amphylaeus morosus	F	1	on *Xanthorrhoea* sp.	*Xanthorrhoea* plus *Dillwynia*	

Table 4.2. Captured putative pollinators in 2002 and 2003 at Site B. Insects carrying orchid pollinaria are indicated in bold type.

Insect Species	**Sex**	**No. Caught**	**Host Plant**	**Pollen Analysis**	**AFLP Analysis**
Apis mellifera (2002)	F	1	*Grevillea sericea*	Legume pollen* (15 grains), plus *G. sericea* (2 grains)	
Leioproctus sp. (2002)	F	1	*Isopogon* sp.	*Isopogon* only	
Exoneura sp. (2003)	F	1	*Diuris alba*	Legume pollen*	

Exoneura sp. (2003)	F	3	*Diuris alba*	no pollen	
***Exoneura* sp. (2003) - with orchid pollinaria**	F	1	*Diuris alba*	*Pultenaea* plus orchid pollen	matches *D. alba*
Exoneura sp. (2003)	F	1	*Diuris alba*	1 *Pultenaea* grain	
***Eurys pulcher* (wasp) (2003) – with orchid pollinarium**		1	*Diuris alba*	orchid pollen	either *D. alba* or *D. aurea***
***Exoneura sp.* (2003) – with orchid pollinarium**	F	1	*Diuris alba*	*Comesperma* plus orchid pollen	matches *D. alba*
Exoneura sp. (2003)	F	1	*Diuris alba*	a few orchid grains	
Paralastor sp. (wasp) (2003)		1	*Diuris alba*	no pollen	

*pollen is likely *Hardenbergia violacea*, but is very similar to *Dillwynia retorta* ** AFLP profile not sufficiently resolved to distinguish these species, which are members of the *Diuris punctata* clade, that is readily distinguished from *D. praecox*.

AFLP Analysis of Orchid Pollinaria

AFLP DNA fingerprinting was able to resolve the distinguishing additional band characteristic for *D. aurea* pollen to be present in the profile for material carried by the honeybee reported in Table 4.1. Absence of this band from bees of two *Exoneura* spp. (see Table 4.2) permitted confirmation of *D. alba* source. However, pollinaria removed from a *Eurys pulcher* wasp (Table 4.2) yielded a profile that was not sufficiently resolved to distinguish *D. alba* and *D. aurea*, but did clearly eliminate *D. praecox*.

UV-Reflectance Photography

Figure 4.3 shows comparative visible light colour, Human Visible Range black and white, and near-UV black and white images for flowers of *Diuris aurea*, *Diuris alba*, *Dillwynia retorta* and *Pimelea linifolia* ssp. *linifolia*. Both *Diuris aurea* and *Dillwynia retorta* reflect near-UV light. In the case of *D. retorta*, a patch at the base of the standard petal forms a nectar guide visible to insects responsive to UV wavelengths. The orchid *D. aurea* has a comparable pattern, similar to that found previously for *D. maculata* (Indsto et al. 2006), including a false nectar guide. We interpret this result as suggestive of legume mimicry for *D. aurea*. By contrast, the UV reflectance of *D. alba*, on the other hand, is dull, and this floral colour component may not be perceived by foraging insects. The white flower has contrasting yellow callous ridges on the labellum, which would be perceived both in the human and insect visual systems. *Pimelea linifolia* ssp. *linifolia*, like many white flowers, does not reflect UV light.

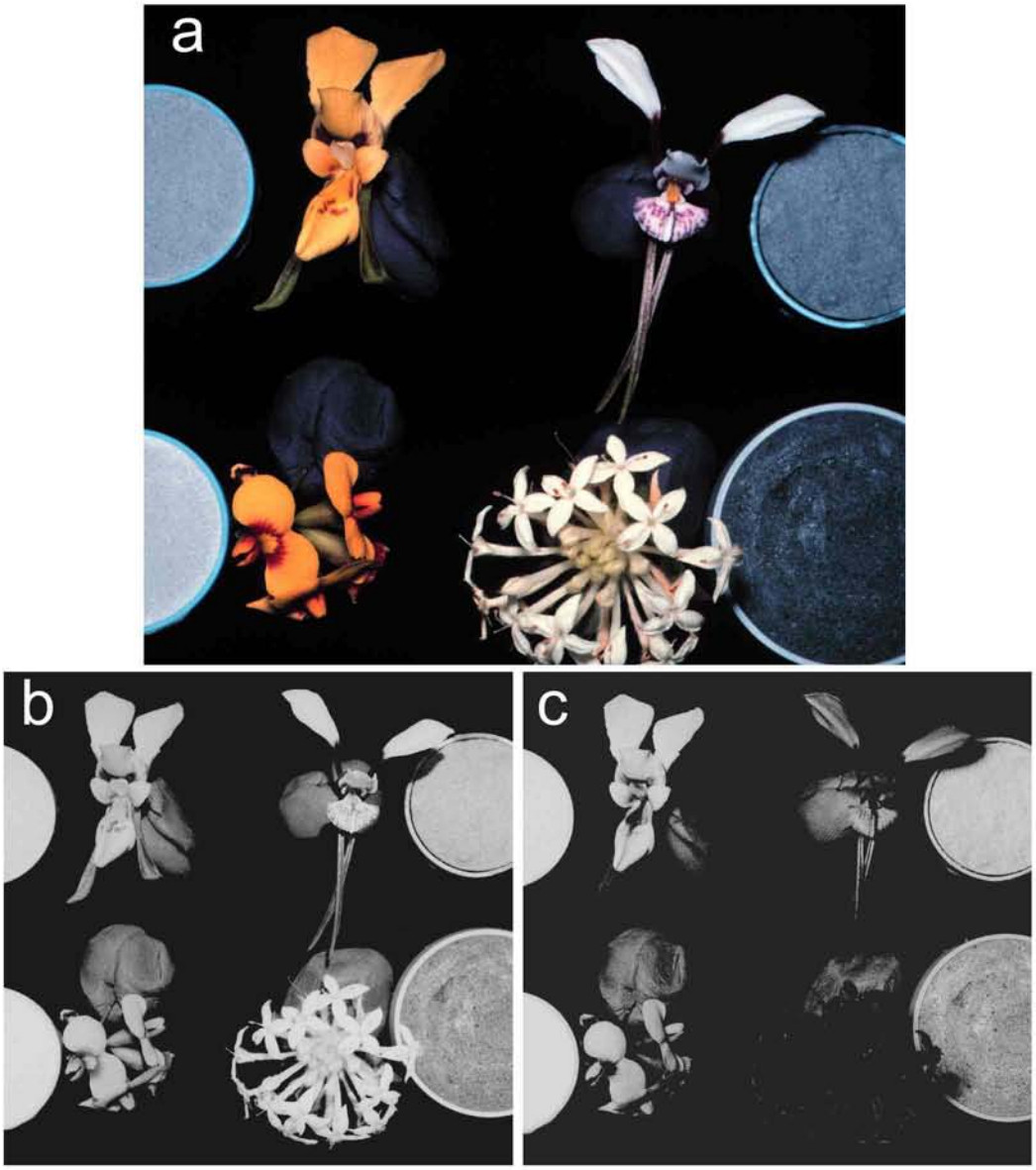

Figure 4.3. Comparative colour and black and white photographic images of *Diuris aurea* (top left), *D. alba* (top right), *Dillwynia retorta* (bottom left) and *Pimelea linifolia* ssp. *linifolia* (bottom right). Reflectance patches positioned in the corners reflect (clockwise from bottom left) 40%. 30% 20% and 10% of incident light, respectively, across the visible and UV ranges, (*a*) Human visible-range (HVR) colour image, (*b*) HVR black and white image, (*c*) Near-UV image. Note: *Diuris alba* flowers are about 25 mm across.

Colorimetric Analysis

Figure 4.4 shows a colour hexagon of flower colours plotted in bee visual space. The three axes of the hexagon correspond to the bee photoreceptor sensitivities in UV, blue and green spectrum components. This shows plots for species found at site A. Locus A (*Diuris aurea*) is quite close in colour to the abundant legume *Dillwynia retorta* (locus C) and is separated by only 0.034 hexagon colour units. The sympatric orchid *Diuris alba* (locus B) is further separated from locus C at 0.08 colour hexagon units. The purple pea *Mirbelia rubiifolia* (locus D) is well separated in bee colour space from either locus A, B or C and is 0.15 hexagon colour units from locus C.

Dyer and Chittka (2004) showed that bumblebees showed increased difficulty in discriminating colours with small colour separations. Translating their findings to this study suggests that a bee would correctly discriminate *Dillwynia retorta* colour from that *Diuris aurea* in only about 60% of choices, whereas a bee would achieve a colour discrimination success between this pea and *Diuris alba* in about 85% of choices. Assuming that bees would not discriminate well between the pea and orchids on the basis of shape and size, *D. aurea* should receive about three times as many visits as *Diuris alba* in the presence of abundant *Dillwynia retorta*. We interpret these results as being compatible with legume mimicry for *D. aurea*. These data suggest that *D. alba* is either not a legume mimic, or a much poorer mimic than *D. aurea*. Interestingly, humans with normal colour vision can distinguish the floral colours of *Dillwynia retorta* and *D. alba* with great ease, but these data suggest an untrained bee would make a colour-based foraging error between these species about 15% of the time.

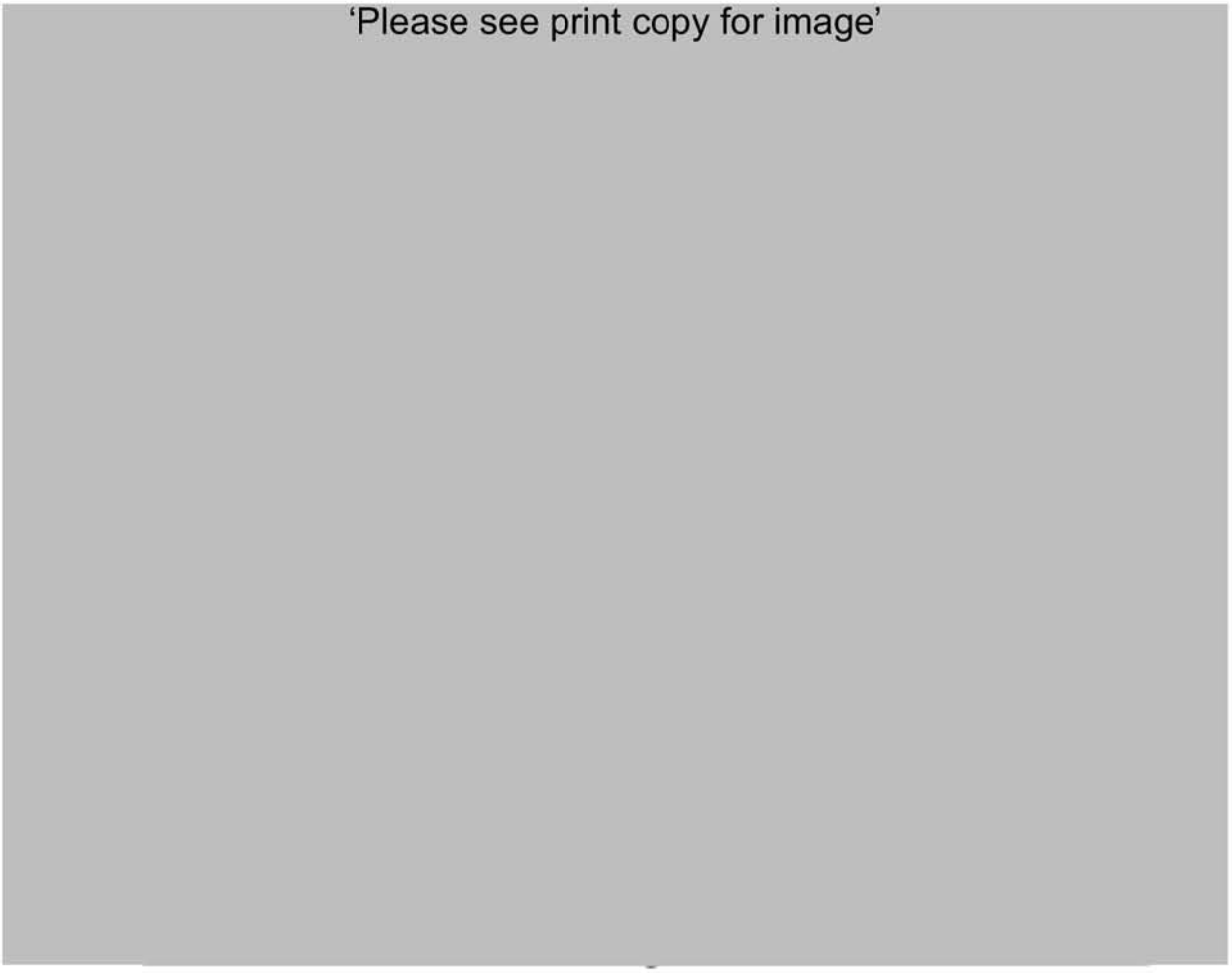

Figure 4.4. Representative species from Site A plotted in a colur hexagon to represent the colour component processing of trichromatic hymenopteran insects (Chittka 1992). In this model, the excitation (E) of the three photoreceptor classes (UV, Blue (B), Green (G) is represented in the corners of the hexagon. The Euclidean distance between loci represents colour similarity as quantified for bumblebee vision by Dyer and Chittka (2004). A, *Diuris aurea*; B, *Diuris alba*; C, *Dillwynia retorta*; D, *Mirbelia rubiifolia*.

Nectar sampling

Eight of ten flowers tested of *Dillwynia retorta* had detectable amounts of nectar, but as the detection method involved addition of distilled water, neither the concentration, nor the quantity could be determined. Similarly, 4 of 15 flowers of *Diuris alba* had very

low, although detectable, amounts of nectar. None of 10 flowers of *Diuris aurea* had any detectable nectar.

Pollination Statistics

Pollinaria removal and fruit set were measured for *Diuris alba* in 2003 at both sites (Table 4.3). *D. alba* at Site A (woodland with abundant *Dillwynia retorta*) had high pollinaria removal (male function) of 73% of flowers and also a high pollinaria deposition (female function, measured as fruit set) of 44%. This compares with statistics for *Diuris aurea* at this site where pollinaria removal of 59% occurred and fruit set was 12%. *D. alba* at Site B (heathy vegetation with uncommon pea flowers) had fewer pollinaria removed (32%), presumably because of fewer pollinators, but relatively high fruit set of 32% of flowers.

Table 4.3. Pollinaria removal data, as a proportion of total flowers open for *Diuris alba* at sites A and B, collected on 19 September 2003. Fruit set data, as a proportion of total flowers are also shown, collected 4-5 weeks after finish of flowering on 17/10/03. Comparative data for *Diuris aurea* at site A is also included.

Site	Pollinaria removal / fruit set
Site A (woodland with *Dillwynia retorta*)	47/64 (73%) / 42/95 (44%)
Site B (grassy heathland)	31/96 (32%) / 49/154 (32%)
Diuris aurea at Site A	16/27 (59%(/5/42 (12%)

Some floral observations

Figure 4.5a shows a recently pollinated flower of *Diuris alba*. The arrow points to the pollen mass. It was rare to find pollinated flowers of this, or other species of Diuris, suggesting that flowers rapidly wither following pollination (a common finding in flowers, including orchids). Flowers of various *Diuris* species, including *D. alba* are often highly variable. This is clearly seen in the array of flowers of different *D. alba* plants shown in Figure 4.5b. Variation in orchid flowers and model pea flowers (where a model is applicable) appears to be the norm. An understanding of the underlying reasons for this variation would no doubt require a project in itself.

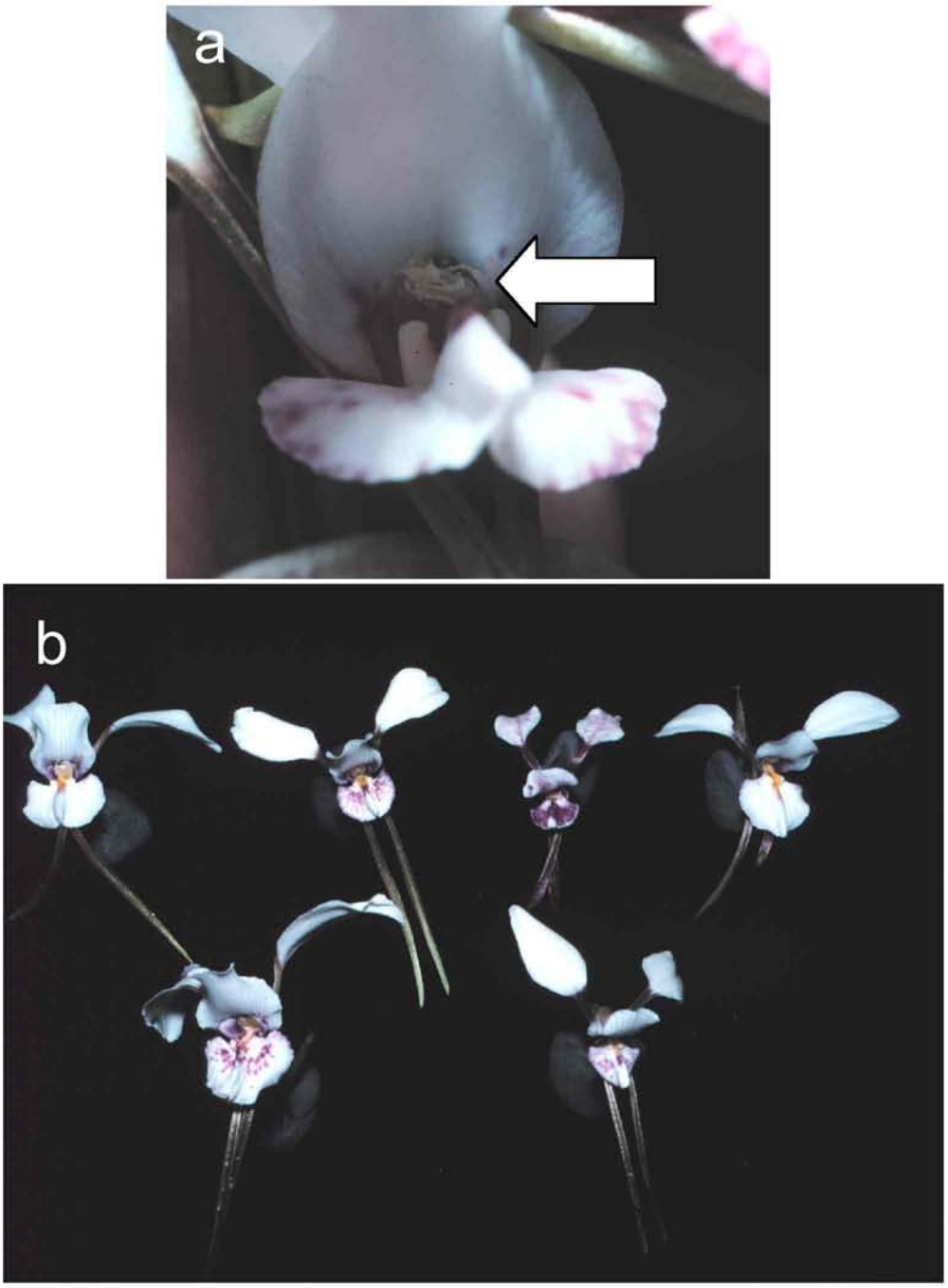

Figure 4.5. Observations of *Diuris alba* flowers. (a) Recently pollinated flower of *D. alba*. The arrow indicates a pollen mass attached to the stigma, (b) Variation in flowers of *D. alba*. A high level of variation appears typical in many *Diuris* species in which populations are formed by seedling recruitment, in contrast to colony forming species which tend to be clonal and highly homogeneous. Note: *Diuris alba* flowers are about 25 mm across.

Discussion

We have shown that small hymenopteran insects, particularly female bees of *Exoneura* spp., are frequent visitors to *D. alba* and are capable of collecting pollinaria. This is strong evidence that these are pollinators of this orchid. Two wasps were also captured visiting *D. alba*. One of these, a Pergid wasp, *Eurys pulcher*, was captured on *D. alba* with orchid pollinaria., the AFLP profile of which was consistent for this species. A wasp from the same genus has previously been reported carrying pollinaria of the terrestrial orchid *Thelymitra antennifera* (Dafni and Calder 1987) and males of this genus show pseudocopulatory behaviour with *Caleana major* (Jones 1988). In studies of suspected orchid mimics, it is common for observations of putative pollinator visits to be rare and, if they are observed, they are brief (e.g. Indsto *et al.* 2006). Our findings of frequent, readily observed, prolonged visits of insects (and their easy capture) to *D. alb*a is strikingly different and suggestive that *D. alba* may be attractive to these insects in its own right, and its colourful, fragrant flowers, with meagre reward, may be attractive to generalist pollinators not specifically seeking out pea flowers.

Dyer and Chittka (2004) showed that bumblebees had increased difficulty in discriminating colours with small colour separations in the bee visual system (Backhaus 1991), and that the likelihood of colour-based errors could be determined according to colour similarity. The spectral sensitivity of hymenopteran insects has been noted to be quite similar (Peitsch et al. 1992; Kevan et al. 2001), thus permitting the extension of their findings to Australian native species. Colorimetric analysis, as might be expected, shows a high similarity in colour between the yellow flowers of the orchid *Diuris aurea*

and the yellow pea *Dillwynia retorta*, including the UV range (this is not a trivial comparison as many flowers which are yellow to human eyes lack a UV colour component and thus would be of very different appearance in the bee visual system). In this case, in the insect visual system, the colour separation was 0.034 colour hexagon units (see Fig. 4.4), which would be expected to result in frequent colour-based foraging errors. This result is consistent with mimicry, as bees are likely to generalise on colours as similar as 0.034 colour hexagon units. The white flowers of *D. alba* are less similar in colour to the pea flowers in the insect visual range, with a colour separation of 0.08 colour hexagon units. However, a colour-based foraging error would still be expected in ~15% of choices. Perhaps surprisingly (to human eyes), a significant number of error-based visits would be expected to sympatric *D. alba* by bees foraging on *Dillwynia retorta*. The purple flowers of the pea *Mirbelia rubiifolia* contrast strongly with the yellow flowers of *D. retorta* and are separated by 0.15 colour hexagon units, a colour distance between stimuli that bees reliably discriminate.

We found the pollination statistics for *Diuris aurea* at Site A (Table 4.3) to be as expected for a pea guild mimic in the presence of an abundant pea model, suggesting not only that this species is a guild mimic, but that its pollination system is highly predictable. By contrast, at this site, *Diuris alba* (which we infer has different pollinators) has a distinctive pollination outcome, especially since fruit set was found to be relatively high in relation to pollinaria removal, compared with *D. aurea*. We suggest the high reproductive success of *D. alba* may be partially explained by the inability of pollinators to consistently distinguish the yellow pea and white orchid flowers, thus resulting in some colour error-based, male function visits. However, the small amount of nectar reward would encourage further visits to orchid flowers, which are sufficiently

distinct in colour from the peas to permit bees to seek them out. It might be expected that *D. alba* has somewhat higher rate of self-fertilisation compared to the non-rewarding *D. aurea* as a consequence of offering a nectar reward (Peter and Johnson 2009). At Site B (heathy site lacking abundant pea flowers), fewer potential pollinators would be expected to be available to visit *D. alba* and lower pollinaria removal (32%, male function) was observed. However, since fewer competing flowers were present, relatively high pollinaria deposition compared with Site A (32%, female function) was observed.

Most orchids in the Diurideae, including *Diuris,* possess soft, friable pollen masses contained in pollinaria that readily fragment, permitting pollination of several consecutive flowers from a single pollinarium (Johnson and Edwards 2000). It is probable that 'male function' may also be less efficient than 'female function' i.e. pollinaria are less likely to be collected in a first visit than collected pollinaria are to be deposited in a second visit. The possibility of an insect pollinating several flowers with one pollinarium would help to explain the similar levels of pollinaria removal and fruit set found for *D. alba* at site B, which would otherwise be possible only if all pollinaria removed were subsequently deposited on other flowers.

Diuris aurea shows a general similarity in floral form and UV visual cues to the legume *Dillwynia retorta,* consistent with mimicry (Fig. 4.3). *Diuris alba* is UV-dull (Fig. 4.3*c*, top right), and reflects much less in the UV range than does *D. aurea* (Fig. 2*c*, top left). However, this reflectance may still be significant and is clearly distinct from the strongly UV-absorbing white flowers of *Pimelea linifolia* ssp. *linifolia* (Fig. 4.3*c*, bottom right). Flowers which appear white to a human observer almost always

lack UV-reflectance. Flowers which are 'insect white' i.e. which reflect more or less evenly across the UV, blue and green parts of the spectrum are rare and would be difficult for bees to see against a background (Kevan et al. 1996).

Pea-flower guild mimicry, which has been proposed for *Diuris maculata s.l.* (Beardsell *et al.* 1986; Indsto *et al.* 2006), has been shown to result in low reproductive success and to involve pollinators that specialise on abundant pea species. Successful pollination would not be expected in the absence of the pea model(s) since the pollinators would also be absent. *Diuris alba* has been shown to be primarily pollinated by the generalist bees of *Exoneura* spp., which show some floral constancy to the pea flower *Dillwynia retorta* when this species is abundant. *D. alba* shows increased reproductive success in the presence of the pea *Dillwynia retorta*, but is also capable of successful pollination where this pea is absent and where sympatric peas are uncommon.

Species of flowering plants may switch from highly specialised to more generalised pollination. This phenomenon has been reported in species of *Dalechampia* (Euphorbiaceae) in Madagascar, which are phylogenetically related to African species pollinated by resin-collecting bees (Armbruster and Baldwin 1998). Dispersal from Africa to Madagascar, where resin-collecting pollinators are absent, has led to the evolution of only pollen as a reward in the open flowers of Madagascan species. As many *Diuris* species are restricted to habitats favouring pea-flower mimicry and pollination by pea specialist bees, they might be expected to be evolutionarily constrained to retain this pollination system. However, in the event of dispersal to habitats favouring generalist pollinators, survival may lead to adaptive changes towards

more generalised pollination. This would appear to be the case for *Diuris alba*, which is phylogenetically related to pea guild mimic species, but appears to have lost some of the features of pea flower mimicry found in a species such as *D. aurea*.

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Chapter 5

A Molecular Phylogenetic Analysis of *Diuris* (Orchidaceae) based on AFLP and ITS reveals three major clades and a basal species

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Prologue

This work has been published in the journal *Australian Systematic Botany:* Indsto, J.O., Weston, P.H, Clements, M.A. (2009) A molecular phylogenetic analysis of *Diuris* (Orchidaceae) reveals three major clades and a basal species. *Australian Systematic Botany* **22**, 1-15. Peter Weston provided considerable input into *Diuris* field collections

and identifications as well as analysis of AFLP and ITS, sequence data processing and cladistic analysis using PAUP and MrBayes computer software. I collected a number of samples within 200 km of Sydney. John Riley and Michael Batley supplied samples from regional New South Wales and interstate. Mark Clements (and David Jones) have worked intensively on *Diuris* for many years. Mark Clements provided many ITS sequences for *Diuris*, which made possible the expansion of analysis to include representatives of all major species groups of this almost exclusively Australian orchid genus. He also identified or confirmed identificaton of many taxa sampled and provided much information on the history of taxonomy in *Diuris*. This is the first phylogenetic analysis of *Diuris*.

Abstract

Diuris is a terrestrial orchid genus of at least 61, and possibly more than 100 species, restricted to Australia except for one species endemic in Timor. Distinctive species groups have respective eastern and western centres of distribution. Although species affinities have been vaguely understood for many years, no formal infrageneric treatment has been undertaken as *Diuris* possess few reliable morphological characters for a classifications system. We have undertaken a cladistic parsimony and Bayesian phylogenetic analysis of *Diuris* using the ITS1-5.8S-ITS2 region of nuclear rDNA, with a subset of samples also studied by amplified fragment length polymorphism (AFLP) as an independent test of phylogenetic relationships. Four major clades with strong bootstrap support were resolved and are named here according to a recently published

classification: *Diuris sulphurea* forms a lineage (subg. *Paradiuris*) of its own that is well supported as the sister to the rest of *Diuris*. Two other major eastern clades contained species related to *Diuris maculata* (subg. *Xanthodiuris*) and *D. punctata* (subg. *Diuris*) respectively. Although these latter two subgenera are genetically well resolved, there is minimal genetic variation at species level, consistent with recent, rapid speciation. A fourth clade (subg. *Hesperodiuris*) has a centre of distribution in Western Australia, and has more genetic and morphological variation than the eastern subgenera. Total evidence analysis provides support for the western clade being sister group to the two eastern subgenera *Diuris* and *Xanthodiuris*, but this relationship was not resolved by molecular data. Hybridisation is known to be common between species within subgenera *Diuris* and *Xanthodiuris*. Instances of incongruence between different data sets were found suggestive of hybridisation events between species of different sections of *Diuris*.

Introduction

According to the compilation of Clements **(http://anbg.gov.au/cpbr/cd-keys/orchidkey/html/currentspecies.html),** the genus *Diuris* (Orchidaceae) comprises at least 61 species that are restricted to Australia, with the exception of *Diuris fryana*, which is endemic to Timor (see Appendix 2). Species of *Diuris* are well represented in the southern parts of Western Australia and eastern Australia, separated by the Nullarbor Plain, with a few species found in tropical Queensland (Jones 1988). The eastern and western species mostly fall into morphologically distinct groups suggestive of distinct phylogenetic lineages. However, a few species that are widely distributed in

south-eastern Australia closely resemble western species (e.g. *D. orientis* cf. *D. corymbosa*). Although some eastern species, such as *D. sulphurea*, or representatives of eastern species groups, extend into South Australia, the centre of diversity of these species groups is eastern New South Wales and, to a lesser extent, Victoria and Tasmania. Many populations and species show considerable variability, making *Diuris* taxonomically challenging at species level. A number of new species have been recognised in recent times and more are likely to be named in the near future.

Dressler (1990) considered, on the basis of an informal phylogenetic analysis of morphological characters, that *Diuris* should be grouped with *Orthoceras*, *Calochilus*, *Epiblema* and *Thelymitra* as the subtribe Diuridinae in the tribe Diurideae. This taxonomic decision was corroborated by subsequent phylogenetic analyses of both chloroplast and nuclear DNA sequences (Kores *et al.* 2001, Clements 2002), although the circumscription of the tribe Diurideae has changed in other respects in response to these molecular results.

Most *Diuris* species show a general resemblance in floral form to Australian legume shrubs, particularly those colloquially known as 'egg and bacon peas', with characteristic yellow base colour and red-brown markings at the base of the standard petal and often other floral parts. These plants belong to a number of genera in the tribes Mirbelieae and Bossiaeeae of the family Fabaceae, including *Pultenaea*, *Daviesia*, *Dillwynia* and *Bossiaea*. The habitats of these orchids are typically open eucalypt forest or woodland communities, which in eastern Australia are extensively distributed in a mosaic pattern that would be expected to result in genetic isolation of many populations (Keith 2004). Most of the literature on pollination of *Diuris* is anecdotal (van der Cingel

2001) apart from 2 studies of the nectarless *Diuris maculata s.l.* (Beardsell *et al.* 1986; Indsto *et al.* 2006). Divergence in floral features from ancestral pea flower mimicry may have occurred in some species e.g. *D. alba* (Indsto *et al.* 2007). These studies provide evidence for Batesian-type floral mimicry of egg and bacon peas with pollination mostly by native bees that specialise in collecting nectar and pollen from pea flowers. As the flowers of the majority of *Diuris* species resemble pea flowers, similarities in pollination mode may be expected in most members of the genus.

A cladistic analysis of *Diuris* species based only on morphology is unlikely to be either well-resolved or well-supported, since the genus provides few taxonomically useful qualitative morphological characters. Moreover, extensive homoplasy in the floral morphology of deceptively pollinated orchids sometimes poses problems for cladistic analyses based predominantly on floral characters (see e.g. (Pridgeon *et al.* 2001). Significant progress in reconstructing the phylogeny of *Diuris* is most likely to be made by sampling a large number of non-morphological characters and including these in a cladistic analysis of the genus. The most cost-effective source of such independent phylogenetic evidence is DNA, sampled either through the determination and alignment of homologous DNA sequences or through the identification of shared, homologous DNA fragments from diverse genetic loci.

The internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA genes (rDNA), ITS1 and ITS2 have proved one of the most informative regions of variable DNA for phylogenetic analysis at the level of species relationships within genera (e.g. (Cox *et al.* 1997; Clements 2003; Orthia *et al.* 2005). The ITS regions are non-coding DNA which is transcribed to RNA, but spliced out during ribosome assembly.

Being non-coding, they accumulate DNA mutations much more rapidly than the 5.8S rDNA gene. Ribosomal genes, although present in high copy number, are usually homogeneous in DNA sequence within individuals through the process of concerted evolution and so are effectively equivalent to the study of variation of a single gene locus (Weider *et al.* 2005). The nuclear genomic ITS1, 5.8S and ITS2 regions of rDNA are contiguous, and being around 700 bases in total length can be readily amplified by PCR as a single unit and studied together. A number of studies which have utilised plastid DNA to provide independent support for analysis of genomic sequences, but have often resulted in conflicting phylogenies (e.g. (Okuyama *et al.* 2004). This has generally been attributable to a consequence of introgression. AFLP (Vos *et al.* 1995) is an alternative technique based on genome-wide DNA sequences containing variable restriction sites. The AFLP technique has the advantage in many cases of distinguishing individuals at intraspecific level (Krauss 2000).

A considerable database of ITS sequences has been lodged with GenBank, but no comparable resource is currently available for AFLP profiles. AFLP has been used to a limited degree in orchid phylogenetic studies (e.g. Hedren *et al.* 2001). AFLP can clearly resolve variable ploidy levels, such as allotetraploidy, in plant lineages. Hedren *et al.* (2001) note that AFLP produces highly reproducible results and that band intensities are highly reproducible. This feature allows AFLP profiles to be readily recognized as patterns or 'fingerprints' (Indsto *et al.* 2005). Patterns of unique bands and altered band intensities are much easier to interpret than polymorphic DNA sequences. We have chosen to include AFLP data to test the findings of ITS sequence analysis because of its value as an independent data set that can potentially reveal incongruence with data from a single locus technique.

Recent publications by Jones (2000) and Jones and Clements (2006) have detailed an infrageneric taxonomy of *Diuris*. These taxon names have been used in this study where supported by our analysis. Whilst morphological characters have been carefully analysed in these publications, unpublished ITS sequence data has also guided the authors in these works. Their total evidence approach as well as research into nomenclatural precedents has resulted in a classification scheme which we test against combined AFLP and ITS data combined with taxonomically informative morphological characters. The data presented here are largely congruent with the taxonomic scheme of Jones and Clements (2006).

Materials and Methods

Plant Samples

Table 5.1 contains information on plant taxa used in this study, including accession details and source localities. Sequences for *Orthoceras strictum* (AF348048) and *Eriochilus cucullatus* (AF348030), were downloaded from GenBank and included in the sample set as outgroup taxa (Clements 2002). An additional sequence for *Diuris sulphurea* (AF348026) was also included from GenBank. Samples for AFLP were collected in the field by JOI, PHW, John Riley (Research Associate, Centre for Plant Biodiversity Studies, CSIRO, ACT) and Michael Batley (Honorary Research Associate, Entomology Department, Australian Museum, Sydney, NSW). Many of these samples were also used for combined ITS1-5.8S-ITS2 (ITS) analysis and combined with

additional samples studied by MAC at the Centre for Plant Biodiversity Studies, CSIRO, ACT.

Table 5.1. Collection details of species samples used in molecular analyses.

Species	Source locality	Collection number	GenBank Accession
Diuris abbreviata	Cathedral Rock National Park, NSW	NSW477113	DQ904011
Diuris aequalis	Kanangra Boyd National Park, NSW	NSW441910	DQ915945
Diuris sp. aff. *alba* 1	Clarence Town, NSW	NSW720057	DQ904027
Diuris sp. aff. *alba* 2	Munmorah, NSW	NSW432988	DQ904012
Diuris alba	Yeppoon, Qld	NSW720059	DQ904020
Diuris arenaria	Nelson Bay, NSW	NSW720050	DQ904028
Diuris aurea 1	Cult. ANBG ex Black Bobs Creek, Hume Hwy, NSW	DW1082	AF348022
Diuris aurea 2	Munmorah, NSW	NSW720114	DQ904013
Diuris aurea 3	Castlereagh Nature Reserve, NSW	NSW719904	AFLP only
Diuris behrii	Stuart Mill Cemetery, Vic	CANB668539	EU595633
Diuris brevifolia	Mt Lofty Range, SA	CANB619900	EU595637
Diuris byronensis	Pattersons Hill, Byron Bay, NSW	CANB648382	EU595641

Diuris carinata	Penelup Farm, Mt Lindsay, WA	CANB668554	EU595626
Diuris chryseopsis	Ilford, NSW	NSW720067	DQ904029
Diuris monticola	Kanangra Boyd National Park, NSW	NSW441907	DQ904019
Diuris concinna	Cult. Ex Helms Arboretum, Gibson, WA	CBG 8506129	EU595630
Diuris conspicillata	Esperance, NSW	NSW720073	DQ904014
Diuris sp. aff. *corymbosa*	1 km S of Marriot, WA	CANB625050	EU595636
Diuris drummondii	Albany, WA	CANB8102383	EU595634
Diuris flavescens	The Bight Cemetery, NSW	NSW720060	DQ904022
Diuris fragrantissima	cult. Ex Tottenham, Vic	CANB940531	EU604809
Diuris goonooensis 1	Conimbla National Park, NSW	CANB732510	EU595638
Diuris goonooensis 2	near Parkes, NSW	NSW720064	DQ904015
Diuris goonooensis 3	Manna Mountain, NSW	NSW720063	DQ904030
Diuris goonooensis 4	Bungambil State Forest, NSW	NSW720071	AFLP only
Diuris goonooensis 5	Reefton, NSW	NSW720077	AFLP only
Diuris goonooensis 6	Round Hill Nature Reserve, NSW	NSW720071	AFLP only
Diuris goonooensis 7	Sims Gap, NSW	NSW720068	AFLP only
Diuris laxiflora	Gordon Crossing, Albany	CANB647853	EU595642

	Hwy, WA		
Diuris maculata 1	Kentlyn, NSW	NSW720115	DQ915944
Diuris maculata 2	Scheyville National Park, NSW	NSW719860	AFLP only
Diuris maculata 3	Lake Parramatta. NSW	NSW720118	AFLP only
Diuris magnifica	Murdoch University, Perth, WA	CANB625020	EU595627
Diuris sp. aff. *ochroma*	Kings Hwy, E of Braidwood, NSW	CANB634123	EU595625
Diuris palustris	Hartley, SA	CANB619672	EU595631
Diuris pardina	Scotts Creek, SA	NSW720075	DQ904023
Diuris picta	Frog Rock, WA	CANB8806717	EU595635
Diuris platichila 1	Dunedoo, NSW	NSW731415	DQ904031
Diuris platichila 2	Halfway between Keith and Pinnaroo, SA	NSW731440	DQ904021
Diuris sp. aff. *porphyrochila* (ms)	Wellesly North Rd, WA	CANB625042	EU595639
Diuris praecox	Bobs Farm, NSW	NSW720061	DQ904016
Diuris punctata 1	Mt Ainsley, ACT	CANB732531	EU595643
Diuris punctata 2	Penrose State Forest, NSW	NSW720113	DQ904017
Diuris punctata 3	Bargo, NSW	NSW720088	DQ904024
Diuris punctata 4	Tallong Cemetery, NSW	NSW720099	AFLP only
Diuris sp. aff. *punctata* 1	Spring Mountain Rd, off Gwyder Hwy, NSW	CANB656698	EU595629

Diuris sp. aff. *punctata* 2	Mellong Swamp, NSW	NSW720039	DQ904032
Diuris semilunulata 1	NSW Southern Tablelands, Braidwood-Nowra Rd	CANB732515	EU595628
Diuris semilunulata 2	near Joadja Creek, NSW	NSW441899	DQ904025
Diuris nigromontana	Black Mountain, ACT	NSW731460	DQ904033
Diuris setacea	E of Cranbourne, WA	CANB668557	EU595632
Diuris sulphurea 1	Wattagan Mountains, NSW	CANB668538	AF348026
Diuris sulphurea 2	Mellong Swamp, NSW	NSW495371	DQ904018
Diuris tricolor 1	Nangerbone State Forest, NSW	NSW720053	DQ904026
Diuris tricolor 2	7 km S of Cowra, NSW	CANB9813821	EU595640
Diuris venosa	Barrington Tops, NSW	CANB668489	EU595644
Eriochilus cucullatus	Mary Seymour Conservation Park, SA	CANB619876	AF348030
Orthoceras strictum	near East Lynne, NSW	CANB 678611	AF348048

Morphological Analysis

For this analysis 10 parsimony-informative morphological characters (see Table 5.2) were coded for all of the sampled species. The data matrix was subjected to cladistic analysis under the Fitch parsimony criterion, using PAUP v. 4.0b for Microsoft Windows (Sinauer Associates, Inc., Publishers, MA, USA). The PAUP analysis consisted of a heuristic search using equally weighted characters and TBR (tree

bisection and reconnection) branch swapping, starting from 1000 trees, each produced from a random addition sequence of taxa. The full morphological data set was then subject to successive approximations character weighting (Farris 1969) using the rescaled consistency index as the measure of fit for new character weights. The morphological analysis was then repeated with the two outgroup taxa, *Eriochilus cucullatus* and *Orthoceras strictum* deleted. This reduced data set was then subject to successive approximations character weighting as above. Strict consensus trees were constructed to summarise the shared components of equally parsimonious trees from all analyses. The morphological dataset was also subject to Bayesian phylogenetic analysis by using the standard discrete model of MrBayes 3.1.2 – one based on the Mk model reviewed by Lewis (2001). MrBayes 3.1.2 spawns two MCMC runs, and we ran each for 4 X 10^6 generations, sampling every 100^{th} generation. A burn-in of 10^6 generations was found to be enough to get the average standard deviation well below 0.01 (a threshold recommended by the authors of MrBayes) for the two runs. For each MCMC run, we constructed a majority-rule consensus of the trees samples after the burn-in period in PAUP* v4.0b10 (Swofford 2003). When the majority rule consensus trees differed between the two runs in the posterior probability of a branch, only the lower of the values is presented.

Table 5.2. Morphological characters used in total evidence analysis

Morphological character	Character states
1. Tuberoid shape	0. Ovoid to ellipsoid; 1. forked ovoid-obovoid; 2. elongate ovoid-obovoid; 3.

	linear-terete
2. Tuberoid orientation	0. Vertical; 1. horizontal
3. Leaf number	0. One; 1. two to three; 2. more than three
4. Colour of predominant anthoxanthin pigments in flower	0. Yellow; 1. white
5. Relative length of lateral sepals and petals	0. Lateral sepals about as long as petals; 1. lateral sepals at least twice as long as petals
6. Lateral sepal shape	0. Linear; 1. linear-spathulate
7. Lateral sepal orientation	0. Semi-erect to erect; 1. horizontal to semi-pendent
8. Relative length of labellum lobes	0. Lateral lobes much shorter than mid-lobe; 1. lateral lobes about as long as mid-lobe
9. Labellum callous	0. With one ridge; 1. with two slightly raised parallel ridges; 2. with two conspicuously raised, divergent ridges
10. Indumentum of labellum callous ridges	0. Glabrous; 1. densely papillate

AFLP analysis

The AFLP procedure of Vos *et al.* (1995) was used with modifications. Fresh whole flowers, or several centimetres of healthy leaf tissue (leaves being thin and grass-like) were dessicated in a resealable bag with silica gel for ~10 days at room temperature and

then stored at -20ºC until required. Dried samples, weighing ~10mg were added to 2ml Eppendorf tubes with a few grains of acid washed sand. Open tubes were placed in 15ml cryovials containing liquid nitrogen to ~20 mm depth and the frozen tissue ground with an autoclaved bamboo skewer. The Qiagen Plant DNeasy Mini Kit (Qiagen GmbH, Germany) protocol was followed without modification and DNA eluted into 200µL AE buffer. The DNA yield was estimated by agarose gel electrophoresis.

AFLP reagents, including restriction enzymes *Eco*R1 and *Mse*1 (New England Biolabs Inc., Beverly, MA) and AFLP adapters and primers (Sigma-Genosys: www.sigma-aldrich.com/life-science/custom-oligos.html) were used as described by Vos *et al.* (1995), except that the *Eco*R1 selective primers were 5'-HEX labelled. A combined restriction digest and ligation was carried out. 200-500 ng of DNA in 10 µL $TE_{0.1}$ ($TE_{0.1}$ = 10mM Tris pH 8.0; 0.1 mM EDTA pH 8.0) was added to 10 µL reaction master mix containing, for 20 µL final volume, 0.5 µM *Eco*R1 adapter and 5 µM *Mse*1 adapter (prepared according to Wolf Lab protocol: http://bioweb.usu.edu/wolf/aflp_protocol.htm), 1 x T4 Ligase Buffer (New England Biolabs), 0.5 µg BSA, 50 mM NaCl, 2 U *Mse*1, 5 U *Eco*R1 and 20 U T4 DNA Ligase (New England Biolabs). The mixture was incubated at 37ºC for 4 h. Ten µL was run on an agarose gel to check for complete digestion (all DNA evident as a visible smear), and the remainder then diluted to 200 µL in $TE_{0.1}$. Four µL of diluted restriction/ligation mix was used as template for pre-selective PCR in 20 µL volumes containing 200 µM dNTPs, 20 ng each of *Eco*R1 and *Mse*1 pre-selective primers, 0.5 µg/µL BSA (Giambernardi *et al.* 1998), 50 mM KCl, 10 mM Tris pH 8.5, 2.5 mM $MgCl_2$, 2% formamide (Ranamukhaarachchi *et al.* 2000) and 1 U *Taq* DNA polymerase. A touchdown PCR protocol was employed with one cycle of 95°C for 3 min, followed by

successive cycles of 95°C for 20 s, annealing for 30 s and 72 °C extension for 2 min, with the first annealing at 66 °C and progressively reduced each cycle by 1° C for the next 10 cycles until an annealing temperature of 56 °C was reached, with 20 cycles carried out at this annealing temperature. This was followed by a final extension step of 72 °C for 10 min. Ten μL was run on an agarose gel to check for a visible smear, indicative of successful amplification and the remainder diluted to 200 μL in $TE_{0.1}$ to serve as template for selective PCR.

Four microlitres of diluted preselective PCR product was used as template for selective PCR. This was carried out with 4 combinations of 2-bp selective primers that were found to be most informative for *Diuris*: *Eco*R1-AC with *Mse*1-CT, *Eco*R1-AA with *Mse*1-CT, *Eco*R1-AA with *Mse*1-CG and *Eco*R1-AC with *Mse*1-CA. *Eco*R1 selective primers were 5'-HEX fluorescently labelled. Reactions were carried out in 20 μL total volumes containing 200 μM dNTPs, 60 ng each of 5'-HEX *Eco*R1-XX and *Mse*1-XX 2-base selective primer pairs, 50 mM KCl, 10 mM Tris pH 8.5, 2.5 mM $MgCl_2$, 0.5 μg/μL BSA, 2% formamide and 1 U *Taq* DNA polymerase and using the same PCR protocol as above, except that 25 cycles of PCR at 56°C annealing were used. An equal volume of denaturing dye containing formamide with 10 mM EDTA pH 8.0 and bromophenol blue was added and the samples heat-denatured for 3 min at 95°C and snap chilled on ice. Aliquots of 2-3 μL were loaded on a 5% 29:1 polyacrylamide gel containing 7.5 M urea and 0.6 X TBE and run in 0.6 X TBE at 40°C and 900 V in a Corbett Gel-Scan 2000 DNA Analyser with He-Ne laser detection.

AFLP chromatograms were printed and compared manually. A *Diuris maculata s.l.* sample was used as the reference taxon in each PCR and gel run and a printout

converted to a transparency. Bands of this species were numbered according to increasing size (gel retention time) on the transparency, which could be overlaid manually on other sample chromatograms. Taxa were then scored for loss of numbered *D. maculata s.l.* bands, or gain of bands, indicated by a letter with position carefully marked in the transparency to 1 base pair accuracy. Each of the four selective AFLP primer combinations listed above generated phylogenetically informative bands. AFLP peaks used in analysis ranged from ~90 to 320 bp, and were each treated as separate characters, were scored for presence or absence and the combined character set was compiled using the software package Nexus Data Editor (NDE Version 5.0, http://taxonomy.zoology.gla.ac.uk/rod/NDE/nde.html).

The AFLP data matrix was subjected to cladistic analysis under the Fitch parsimony criterion, as for morphological data, and to Bayesian phylogenetic analysis, by using MrBayes 3.1.2. The PAUP analysis consisted of a heuristic search using equally weighted characters and TBR (tree bisection and reconnection) branch swapping, starting from 1000 trees, each produced from a random addition sequence of taxa. Bootstrap analysis with 2000 replicates was conducted using default heuristic search settings, generating a majority rule consensus tree of nodes with > 50% BP support. Two MrBayes analyses were conducted, one using the restriction site (binary) model with no absence sites, and the other with the standard discrete model. For the first analysis, two MCMC chains were run for 6 X 10^6 generations, sampling every 100^{th} generation with a burn-in of 7.5 X 10^5 generations. Majority-rule consensus trees were constructed from the trees sampled after the burn-in period in each MCMC run, as in the morphological analysis.

A total of 31 taxa, including 30 ingroup taxa were studied for the total of 48 AFLP characters, of which 20 were parsimony-informative. The AFLP profiles of the chosen outgroup taxa differed so much from *Diuris* that homologous bands could not be confidently identified, so these taxa could not be usefully included in the analysis. Consequently, unrooted trees were produced and re-rooted on the branch connecting *Diuris sulphurea* to the rest of the genus, in order to render the results readily comparable to those of our analysis of ITS sequences (see below).

Combined ITS1-5.8S-ITS2 rDNA (ITS) Plus indels analysis

The contiguous ITS1-5.8S-ITS2 rDNA (referred to as 'ITS' hereafter) region was amplified as one unit using mainly the primer pair 17SE and 26SE (Sun 1994; Gravendeel 2001), but other combinations were also used (Clements 2002). PCR reactions were performed using 20 ng sample DNA in 50 μL volumes containing 10 mM Tris pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μg/μL BSA (Giambernardi *et al.* 1998), 150 ng each primer and 3 U *Taq* DNA polymerase. The PCR protocol was one cycle of 95°C for 3 min, followed by 25 cycles of 95°C for 20 s, 58°C for 30 s and 72°C for 2 min and followed by a final extension of 72°C for 10 min. PCR product yield was checked by agarose gel electrophoresis and purified for sequencing using the Qiagen QiaQuick kit (QIAGEN). PCR products of approximately 850 bp length were bi-directionally sequenced using Applied Biosystems Big DyeTerminator v3.0 chemistry and run on an Applied Biosystems ABI Prism® 3100 Genetic Analyser.

DNA sequence chromatograms were edited using Sequencher 3.0 software (Gene Codes Corporation) or the software package Finch-TV (Geospiza, Seattle, WA, USA). A text file of all taxa (50 in total, 48 ingroup taxa) in FASTA format was assembled and an alignment analysis performed using the software package Clustal X (Bio Pack 3.6, including also BioEdit and Treeview; Ibis Biosciences, Carlsbad, CA, USA), with default parameter settings. The aligned sequences were further processed to remove flanking DNA sequence using BioEdit, and then exported in Nexus format for analysis in PAUP v. 4.0 as detailed above for morphology and AFLP, except that the trees were rooted using the designated outgroups, branch swapping started from 200 trees, each produced from a random addition sequence of taxa, maxtrees was set at 100, and only 1000 bootstrap replicates were completed. The analysis comprised 715 total characters, of which 122 were parsimony informative. Eleven indels were coded as additional characters by the simple indel-coding method of Simmons and Ochotorena (2000).

The ITS dataset was also subject to Bayesian phylogenetic analysis with MrBayes 3.1.2. We used the Akaike information criterion (AIC) in MrModelTest 2.3 (available from J.A.A. Nylander's website at http://www.abc.se/~nylander/mrmodeltst2/mrmodeltest2.html) to select an adequately parameter-rich model of nucleotide substitution for the ITS alignment, with indels coded as missing data. Indels were then added to the data matrix as an extra partition of binary characters (as for the parsimony analysis) and were analysed by using the standard discrete evolutionary model. We unlinked sampling of state frequencies and substitution rates for the two data DNA partitions. The two MCMC runs were run for 3 X 10^6 generations, sampling every 100^{th} generation. We found that a burn-in of 7.5 X 10^3

generations was sufficient to get the average standard deviation of split frequencies well below 0.01 for the two runs. Majority-rule consensus trees were constructed from the trees sampled after after the burn-in period in each MCMC run, as in the morphological analysis.

Total Evidence Analysis

For this analysis all morphological, AFLP and ITS characters were combined and analysed as a single data set as described for the ITS data set above. The combined dataset was also subject to two Bayesian phylogenetic analyses with MrBayes 3.1.2, treating the ITS nucleotide sites, ITS indels, AFLP data and morphological characters as four partitions and unlinking the sampling of state frequencies and substitution rates for each of the four data partitions. The two analyses differed in the model used for the AFLP partition; in the first we used the restriction-site (binary) model with no absence sites, whereas in the second the standard discrete model was used. The models used for the other three partitions were the same as those used in the separate analysis of these partitions. We ran the first MCMC analysis for 6 X 10^6 generations, and the second for 4 X 10^6 generations, sampling every 100^{th} generation in both cases. In the analysis that used the restriction-site (binary) model with no absence sites, the average standard deviation had reduced to only 0.012 after 6 X 10^6 generations, so, strictly speaking, it had not yet emerged from its burn-in phase. We concluded that this mixed model was intractably complex and terminated the analysis. By contrast, in the analysis that used the standard discrete model for the AFLP partition, the average standard deviation of split frequencies reduced to below 0.01 after 6.87 X 10^5 generations; thus, discarding the first 10^6 generations as the burn-in was more than adequate. Majority-rule consensus

trees were constructed from the trees sampled after the burn-in period in each of the two MCMC runs of the analysis, by using the standard discrete model for AFLP data, as in the morphological analysis.

Morphological-character phylogenies were reconstructed by mapping characters parsimoniously on the total evidence trees using the 'trace character history' option in Mesquite version 2.01 (build j28; http://mesquiteproject.org).

Results

The parsimony analysis including all sampled species produced 402 equally parsimonious trees of length 19 steps. The strict consensus of these trees (not shown) included only one resolved component, a grouping of all five of the sampled species of *Diuris* subgenus *Diuris* section *Pedunculatae*. The rest of *Diuris* and its outgroups formed a polytomy. Only 248 trees of length 13.61572 were produced using successive approximations character weighting but these yielded the same consensus tree as for the unweighted data. The two outgroup taxa, which were both scored as 'unknown' for several characters, were then deleted and the remaining taxa reanalysed as before, to test whether the presence of unknown cells in the data matrix was responsible for the observed lack of phylogenetic resolution. This resulted in 6744 trees of length 18 steps for unweighted data and 3228 trees of length 14.81667 steps for weighted data. The strict consensuses of both sets of trees (not shown) were identical and included only one resolved component, the same as in the previous results produced with the full sample of species. The two majority-rule consensus trees from the Bayesian analysis had three clades resolved but two of these received posterior probabilities of less than 0.95. The

clade corresponding to *Diuris* section *Suffusae* (Appendix 2) received a posterior probability of 0.95.

The parsimony analysis of the AFLP dataset produced one tree of length 39 steps (see Figure 5.1). This clearly resolved the subgenera *Diuris* and *Xanthodiuris sensu* Jones and Clements (2006) with strong bootstrap support of 100 and 94 respectively. According to AFLP these subgenera form a clade with BP = 93 and are more closely related to each other than to the subgenera *Hesperodiuris* and *Paradiuris,* which together form an unresolved basal grade. Species within subg. *Diuris*, sect. *Diuris*, comprising species with yellow pigments as the dominant anthoxanthins in their flower are resolved by just one AFLP band from species in sect. *Purpureo-albae*, which have white pigments as the dominant anthoxanthins in their flowers (BP = 66). Sections within subg. *Xanthodiuris* are poorly resolved in AFLP analysis. The majority-rule consensus trees from the two Bayesian analyses of the AFLP dataset had identical topologies, despite the different evolutionary models that were used to produce them. They were also almost identical to the parsimony tree (see Fig. 5.1 for posterior probabilities), differing only in not resolving *D. monticola* as sister to the rest of subg. Xanthodiuris. However, the clades corresponding to *Diuris* subgenera *Diuris* plus *Xanthodiuris* received significant posterior probabilities, i.e. 0.95 in the analysis with the standard model and 0.97 with the restriction-site (binary) model with no absence sites.

The alignment of ITS sequences plus coded ITS indels, which consisted of 704 putatively homologous nucleotide positions for 50 taxa, plus 11 indel characters, provided 239 variable characters of which 122 were parsimony-informative. The heuristic parsimony search using this data set was stopped when >1.9 million trees of

length 376 steps had been found. One of these is shown in Figure 5.2. The 50% majority rule bootstrap consensus tree is shown in Figure 5.3. *Diuris sulphurea* (subg. *Paradiuris*) forms a sister group to the rest of *Diuris* (BP = 100). The subgenera *Diuris*, *Hesperodiuris* and *Xanthodiuris* form 3 well-supported clades (BP = 100) with similar levels of genetic divergence from each other. However, within subg. *Diuris*, just one DNA base difference resolves sect. *Diuris* from sect. *Purpureo-albae*. Similarly in subg. *Xanthodiuris*, one DNA base separates sect. *Abbreviatae* from sect. *Xanthodiuris*. Sect. *Pedunculatae* contains two DNA base differences from sect. *Xanthodiuris*; however, owing to polymorphisms suggestive of hybridisation in two species samples, this section has only 48% bootstrap support and hence is not resolved on this tree. By contrast species within subg. *Hesperodiuris* show much greater genetic diversity. Section *Suffusae* (BP = 99) is well supported as circumscribed. Section *Hesperodiuris* shows a relatively high level of genetic heterogeneity with two well-supported internal clades represented by *Diuris drummondii* (BP = 99) and *D. setacea* (BP = 87). Section *Hesperodiuris* can be considered well supported, although the internal relationships of this section as represented in the consensus tree are only moderately supported (BP = 66). The majority-rule consensus trees from the Bayesian analysis of the ITS dataset were very similar to the bootstrap consensus tree from the parsimony analysis

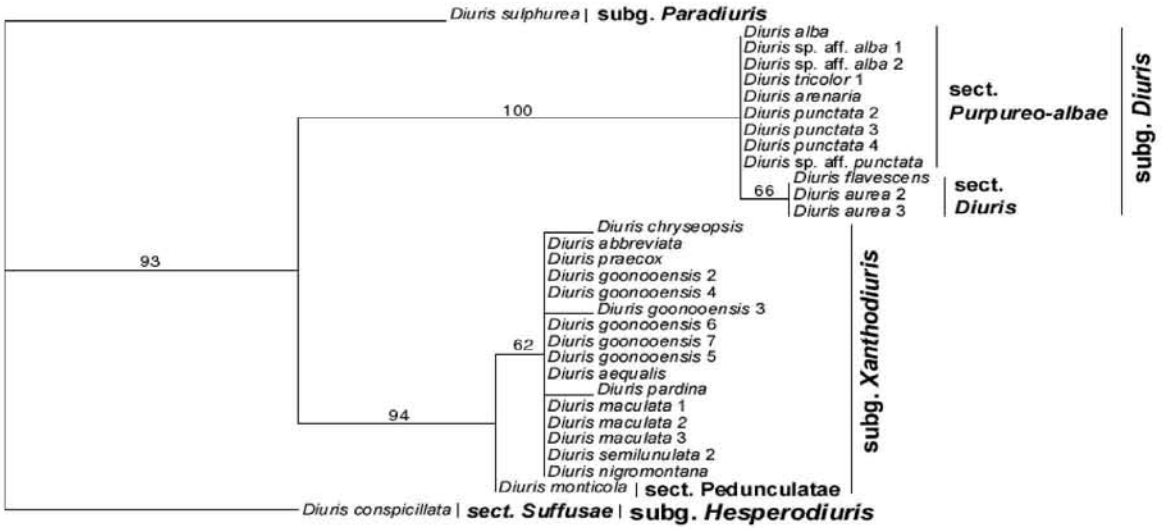

Figure 5.1. The one tree of length 39 steps produced by parsimony analysis of AFLP data, showing branch lengths estimated under the ACCTRAN algorithm, and annotated with bootstrap support indices. A total of 31 taxa, including 30 ingroup taxa were studied for 48 AFLP characters, of which 20 were parsimony informative.

(see Fig. 5.3 for posterior probabilities), resolving only two of these clades with posterior probabilities of <0.95.

Trees for AFLP and ITS are highly congruent so far as resolution allows comparison. We expected that AFLP would produce a more highly resolved tree than ITS since other studies had found AFLP to be sensitive enough even to resolve the parentage of plants in a population (Krauss 2000). For a given primer combination the number of AFLP bands is expected to be proportional to genome size (Vos *et al.* 1995). As it was necessary to use 2-bp selective primers to obtain sufficient bands for analysis, we expect that the genome of *Diuris* is relatively small.

The analysis of the combined dataset had to be stopped when it had produced more than 1.6 million trees of length 424 steps. The results are summarised in Fig. 5.4, the bootstrap consensus tree, which is more highly resolved than those produced from any of the data partitions. The total evidence tree shows moderate support (BP = 74) for the mainly western Australian subgenus *Hesperodiuris* being a sister group to the eastern subgenera *Diuris* and *Xanthodiuris*. Within subg. *Hesperodiuris*, *Diuris palustris* of the monotypic section *Palustres* appears as a sister group to sect. *Hesperodiuris* only, rather than sister to the whole of subg. *Hesperodiuris* as found in the ITS data. Within subg. *Diuris*, *Diuris tricolor* has equivocal placement in the total evidence tree as the floral characteristics are intermediate between sections *Diuris* and *Purpureo-albae* (yellow floral pigments of sect. *Diuris* with elongated lateral sepals of sect. *Purpureo-albae*). In addition, AFLP data suggest this species has affinity with sect. *Purpureo-albae*, while ITS evidence places it with sect. *Diuris*. Within subg. *Xanthodiuris*, sect. *Pedunculatae* is moderately well supported in the total evidence tree (BP = 82) as these

species show clear morphological distinctions with sect. *Xanthodiuris* such as drooping lateral petals, densely papillate-hirsute callus ridges, and higher leaf number. The majority-rule consensus trees from the Bayesian analysis of the combined dataset were very similar to the bootstrap consensus obtained with maximum parsimony (see Fig. 5.4 for posterior probabilities). Six clades were resolved differently by the different analyses; however, all of these involved branches with posterior probabilities <0.95.

Discussion

As we had expected, the results of our analyses of the morphological data set were very indecisive, consistently producing only one putative clade, composed of all five of the sampled species of *Diuris* subgenus *Diuris* section *Pedunculatae*. This confirmed the need for additional evidence before the phylogeny of *Diuris* could be reconstructed with accuracy and precision. Analysis of two independent DNA data sets, ITS rDNA sequences and AFLP resulted in highly congruent cladograms, both of which contained several strongly supported clades. This confirmed the wisdom of sampling molecular characters to supplement the morphological data set. ITS analysis strongly supported the monophyly of *Diuris* and a basal split between *Diuris sulphurea*, the only species in *Diuris* subgenus *Paradiuris*, and a clade containing all other sampled species. Within the latter clade, both molecular data sets produced groupings corresponding to the remaining three subgenera of Jones and Clements (2006), although one of these, subgenus *Hesperodiuris*, was represented by only one species, *D. conspicillata*. The AFLP analysis strongly supported a sister group relationship between subgenera *Diuris* and *Xanthodiuris* in contrast to the ITS analysis, which could

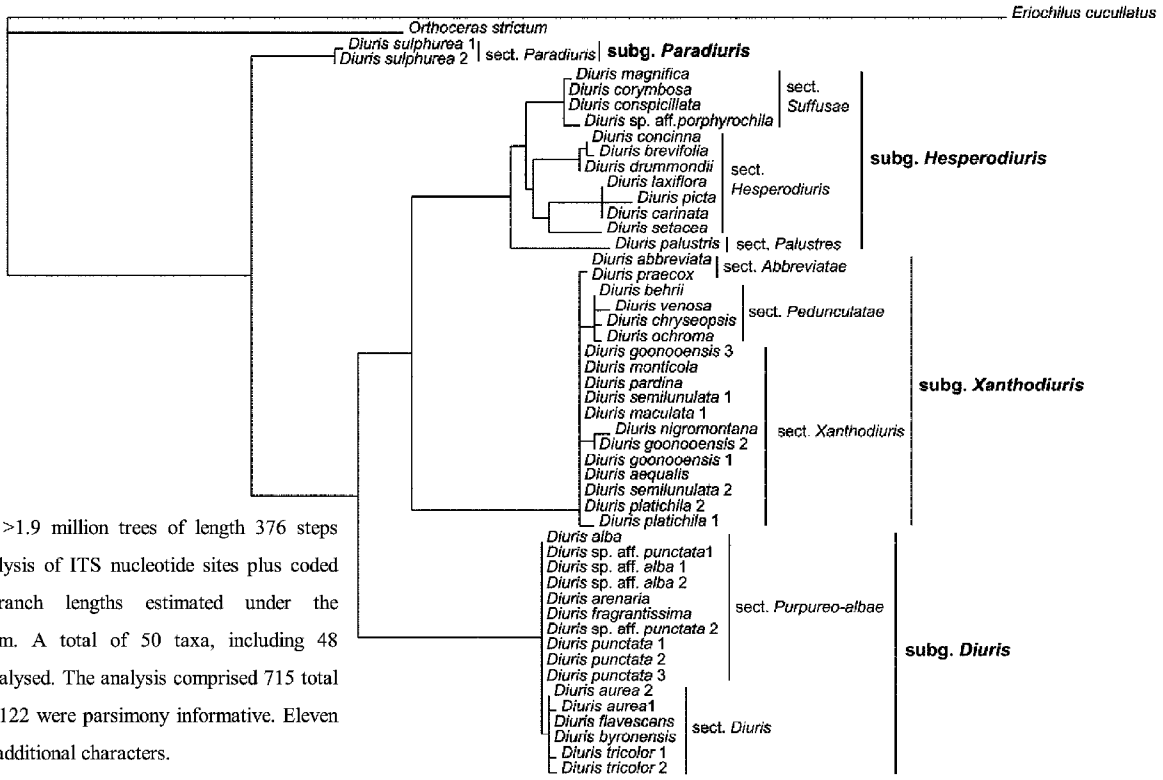

Figure 5.2. One of >1.9 million trees of length 376 steps produced by the analysis of ITS nucleotide sites plus coded indels, showing branch lengths estimated under the ACCTRAN algorithm. A total of 50 taxa, including 48 ingroup taxa were analysed. The analysis comprised 715 total characters, of which 122 were parsimony informative. Eleven indels were coded as additional characters.

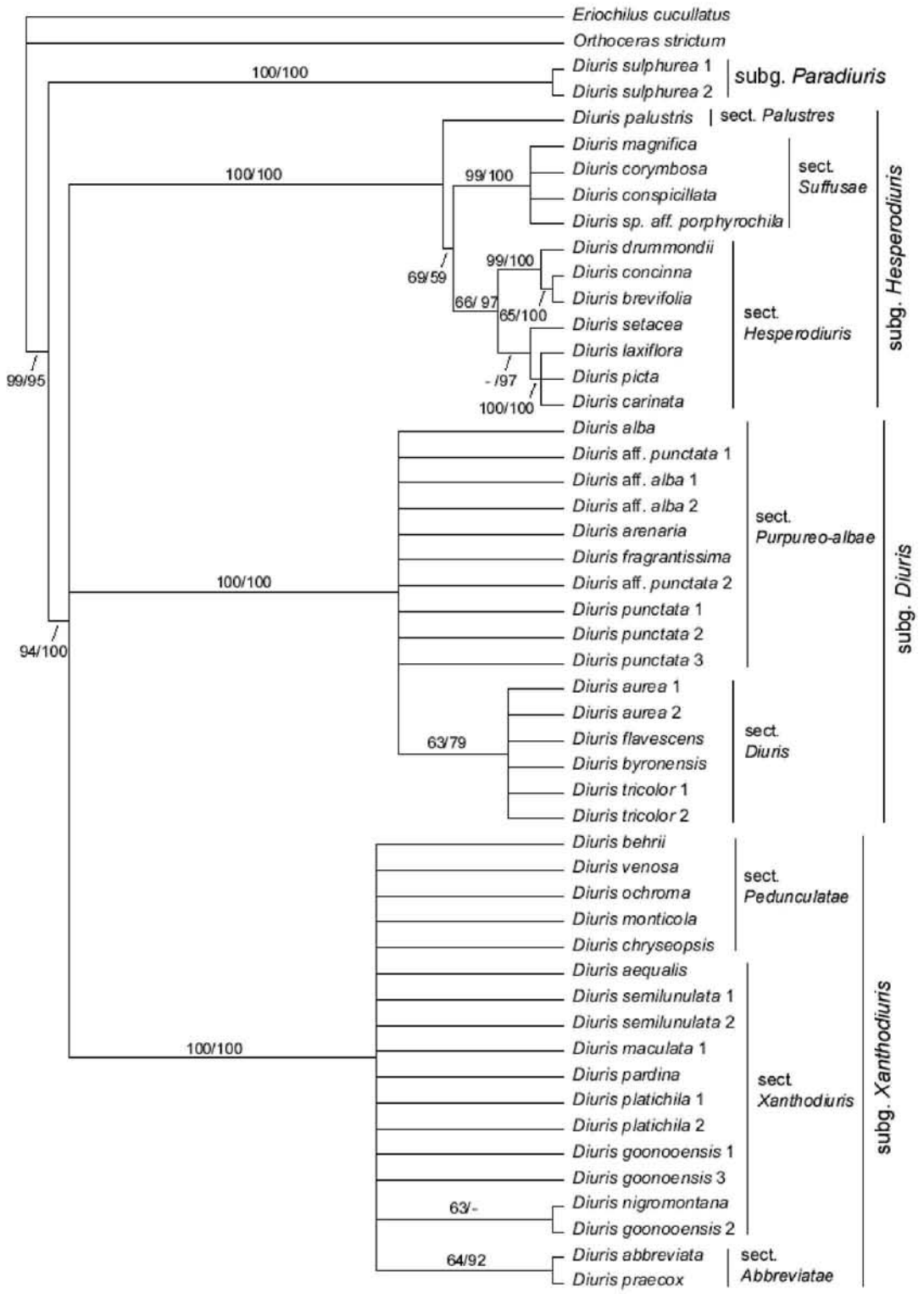

Figure 5.3. Bootstrap 50% majority rule consensus tree for a total of 50 terminals, produced by analysis of ITS nucleotide sites plus coded indels, which summarises >1.9 million equally parsimonious of length 376 steps. Above each branch are its bootstrap support index (left of the slash) and Bayesian posterior probability (right of the slash), both expressed as percentages. The analysis comprised 715 total characters, of which 122 were parsimony informative. Eleven indels were coded as additional characters.

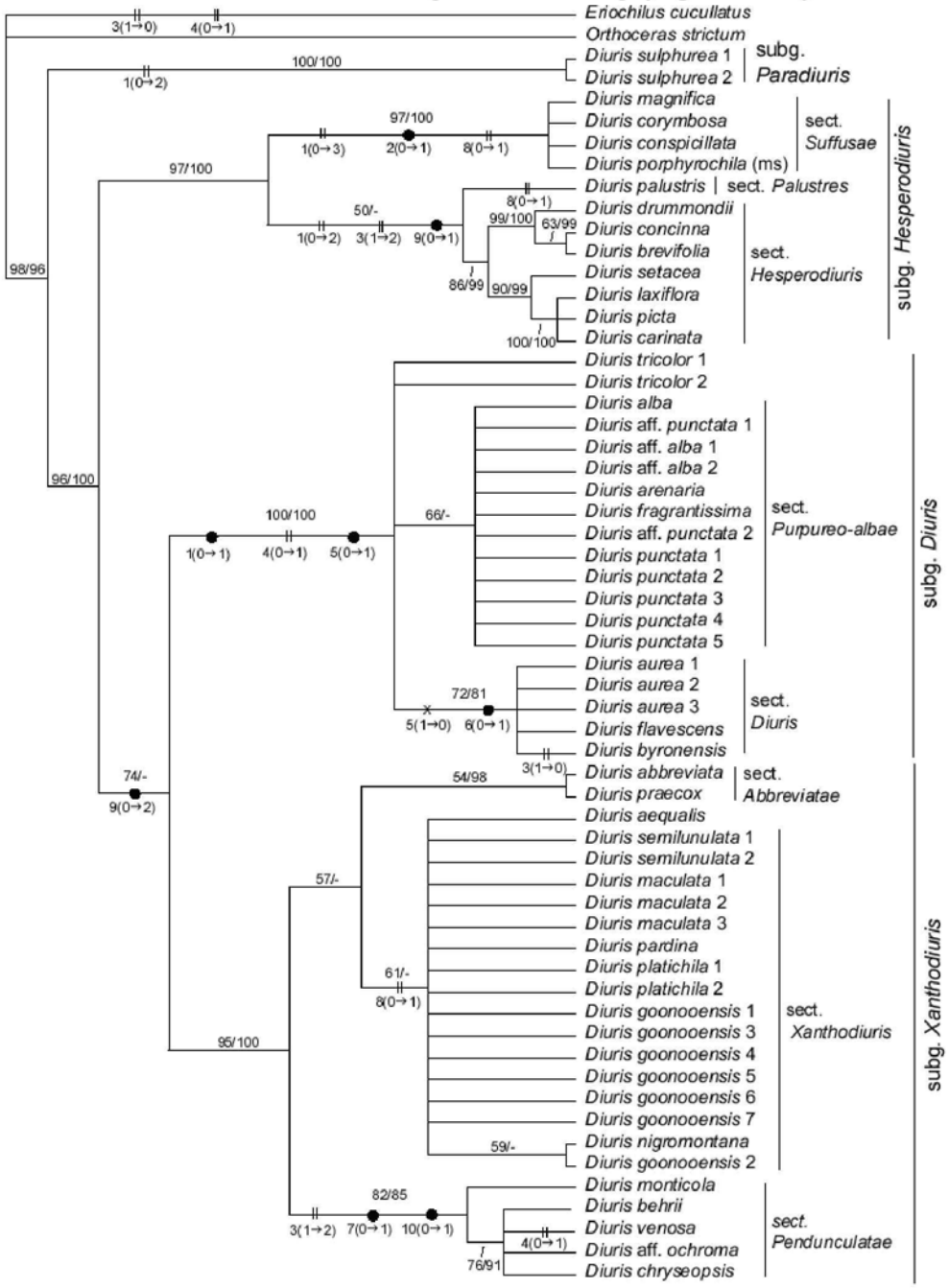

Figure 5.4. Bootstrap 50% consensus tree for a total of 59 terminals, produced by analysis of a combined dataset of all sampled characters, which summarises >1.6 million equally parsimonious trees of length 424 steps. Datasets for AFLP, ITS sequence plus indels, and 10 morphological characters were combined and analysed as a single dataset. Above each branch are its bootstrap support index (left of the slash) and Bayesian posterior probability (right of the slash), both expressed as percentages. The marks on the branches refer to morphological character-state changes listed below the marks, reconstructed by using Fitch parsimony (● denotes a unique forward change); ‖ specifies a parallel forward change, x denotes a reversal. Note that the character phylogeny for Character 8 is one of two equally parsimomious reconstructions.

not resolve relationships between these clades and subgenus *Hesperodiuris*. Interestingly, neither of the molecular data sets resolved the only clade that emerged from analyses of morphological characters, *Diuris* subgenus *Diuris* section *Pedunculatae*, which formed part of a larger polytomy in the ITS analysis and was weakly resolved as paraphyletic in the AFLP analysis. All three data sets therefore provided potentially complementary information on the phylogeny of *Diuris*.

The total evidence analysis of all three data sets provided the most highly resolved, best supported bootstrap consensus tree of all of our analyses, even though it yielded such a large number of equally parsimonious trees that it needed to be stopped before completion of branch swapping. It also yielded the most highly resolved, best-supported Bayesian probability tree, which was highly congruent with the parsimony tree. These analyses supported the monophyly of all of the subgenera and all but two of the sections that had been proposed by Jones and Clements (2006). However, *Diuris* section *Setaceae* D.L.Jones & M.A.Clements was found to be embedded within section *Hesperodiuris* and, hence, is not supported by the present study. Species within *Diuris* section *Pyrophilae* D.L.Jones & M.A.Clements were not studied in the present analysis.

To the extent that resolution allowed, ITS and AFLP data sets produced highly congruent cladograms. This confirmed the efficacy of the technique proposed by Indsto *et al.* (2005) of using AFLP profiles to identify pollinaria remnants collected from the bodies of putative pollinators. Surprisingly, the AFLP data were somewhat less phylogenetically informative than ITS data, which showed slightly higher resolution of inter-specific relationships. Even more surprisingly, the AFLP data showed almost no infraspecific variation across all sampled *Diuris* species within sections, in contrast to

previous studies of other taxa, in which AFLP had been used at the intraspecific level for purposes demanding highly variable markers, such as paternity analysis (Krauss 2000; Coyle *et al.* 2003). A simplistic response to this result would be to suggest that in eastern Australia, species of *Diuris* have been circumscribed too narrowly and that species boundaries should be re-drawn at the limit of molecular phylogenetic resolution, if not higher. If implemented, this would reduce the number of eastern Australian species in our sample from 27 to four or fewer. This response, however, would be a mistake. Firstly, it would treat as conspecific several entities that are both morphologically diagnosable and broadly sympatric and, in some cases, adapted to growing in distinctly different habitats. For example, *Diuris chryseopsisi* and *D. pardina* would be treated as conspecific, even though they are broadly sympatric in southern New South Wales, Victoria, South Australia and Tasmania, and frequently co-occur but hybridise only sporadically. Secondly, AFLP variation is surprisingly low even within broadly defined species groups so it seems unlikely to be an artifact of excessive taxonomic 'splitting'. Since AFLP provides dominant markers, it is unable to yield estimates of heterozygosity; however, it would be interesting to use a co-dominant marker system to test whether the low level of AFLP variation found in this study reflects a generally low level of genetic variation, or just a small nuclear genome.

Hybrids between species within the eastern subgenera *Diuris* and *Xanthodiuris* are well known. For example, in subgenus *Diuris*, *D. aurea* (sect. *Diuris*) and *D. punctata* (sect. *Purpureo-albae*) are sometimes sympatric and form the hybrid *D.* x *nebulosa*, which is intermediate in floral form between these species and readily identifiable in the field. Hybrids have also been commonly observed between species within subgenus *Xanthodiuris* particularly sections *Xanthodiuris* and sect.

Pedunculatae. Hybrids between species of different subgenera are rare. The frequency of natural hybridisation observed between sympatric species fits well with expectations based on the molecular distances between species presented here.

A couple of instances of incongruence between data sets in the placement of individual species are presumably the results of either past hybridisation or incomplete lineage sorting. One of these involves the phylogenetic position of *Diuris tricolor*, the only species that has both elongated lateral sepals typical of section *Purpureo-albae* and yellow pigments as its dominant floral anthoxanthins. These morphological characters are incongruent with the ITS tree in which *Diuris tricolor* forms part of *Diuris* subgenus *Diuris* section *Diuris*, a yellow-flowered clade that mostly has short lateral sepals. The AFLP result is potentially congruent with either ITS or morphology because it places *Diuris tricolor* as part of a large polytomy with other species of section *Purpureo-albae* plus section *Diuris*. The observed patterns of character distribution are consistent with *Diuris tricolor* having evolved through hybridisation between ancestral species that belonged to sections *Purpureo-albae* plus section *Diuris*. Alternatively, if *Diuris tricolor* really is the sister species of section *Purpureo-albae*, its anomalous ITS sequence could be explained by retention of an ancestral ITS polymorphism in the most recent common ancestor of these taxa, followed by fixation of alternative alleles in its immediate descendants.

The parsimony tree for AFLP tree is incongruent with morphology with respect to the placement of *Diuris monticola*. It placed this species as sister to the rest of sections *Xanthodiuris*, *Abbreviatae* and *Pedunculatae*, whereas morphology alone placed this species in the only putative clade that it decisively resolved, section

Pedunculatae. The ITS results are potentially congruent with either AFLP or morphology because it includes all of the species of sections *Pedunculatae* and *Xanthodiuris* in one large polytomy with section *Abbreviatae*. These observed patterns of character distribution are consistent with either hybridisation or lineage sorting processes, as described above for *D. tricolor*.

Of the ten morphological characters that we included in our analyses, five are completely consistent with the consensus tree derived from our parsimony analysis of the combined dataset. Four of these provide synapomorphies for small species groups whereas only one, labellum callus, was reconstructed as changing state near the base of the tree. A labellum callus with one ridge was resolved as the ancestral condition in *Diuris*, which subsequently transformed to one with two slightly raised parallel ridges in the clade comprised of sections *Hesperodiuris sensu lato* and *Palustres* and to one with two conspicuously raised, divergent ridges in subgenus *Diuris*. The phylogenetic signal of this character has been overwhelmed by the conflicting signals provided by the five homoplasious characters in the morphological analysis. Other clades marked by synapomorphies that were consistent with the total evidence analysis were: section *Suffusae* (horizontal tuber orientation), section *Diuris* (lateral sepals linear-spathulate) and section *Pedunculatae* (lateral petals horizontal to semi-pendent; labellum callus ridges densely papillate). Within subgenus *Xanthodiuris*, leaf number is distinctly higher in section. *Pedunculatae* than in sections *Xanthodiuris* and *Abbreviatae* (>3 cf. 1-2 in sections *Xanthodiuris* and *Abbreviatae*), but this feature also evolved in parallel in section *Hesperodiuris*. Within subgenus *Diuris*, distinctive forked tubers are found but their presence is polymorphic in some species. Species within sect. *Purpureo-albae* have white floral anthoxanthins and long lateral sepals, both of which appear to be

synapomorphies for this section. Within *Diuris* subgenus *Xanthodiuris* section *Pedunculatae* there are some species such as *D. venosa* which also have white floral anthoxanthins and this appears to have arisen independently in these species. Long lateral sepals comparable to those of section *Diuris* are also found in *Diuris tricolor* but, as discussed above, this character maps equivocally on our best estimate of phylogenetic relationships. The large lateral labellum lobes of section *Xanthodiuris* are a conspicuous synapomorphy for this clade, but this is also a feature of section *Suffusae*.

What can be inferred about the age of *Diuris* and evolution over time? Molecular dating analysis cannot yet provide us with a calibrated chronology because we have no fossils of *Diuris* to provide age contraints and very few for the Orchidaceae as a whole (Ramirez *et al.* 2007). However, inspection of the distribution of relative branch lengths in our molecular trees reveals some striking contrasts in macroevolutionary pattern. Within the predominantly Western Australian subgenus *Hesperodiuris* a number of clades are well-resolved according to ITS data and sections within this subgenus have strong bootstrap support. The shape of the ITS phylogram for this subgenus appears to be consistent with approximately constant rates of diversification. In contrast, within the predominantly south eastern subgenera *Diuris* and *Xanthodiuris*, there is minimal DNA evidence to distinguish species and to provide support for sections, a pattern that is also shown in the AFLP phylogram. The shape of the molecular phylograms for these clades resembles a pair of rakes, with long, unbranched 'handles' and broad 'heads' bearing short 'tines'. This suggests that the macroevolutionary processes that have shaped the phylogeny of *Diuris* have differed significantly on either side of the Nullarbor Plain. The Western Australian taxa seem to have evolved by a process of phyletic gradualism

whereas evolution of the south eastern clades seems to have involved either high rates of extinction or very sporadic rates of speciation, or both, as would be expected under the model of punctuated equilibria (Gould and Eldredge 1972). The latter model proposes that rapid species radiation follows disturbance and species extinctions, or evolutionary innovation (Gould and Eldredge 1972). This rapid change is then followed by periods of relative stasis. The contrasting patterns observed in *Diuris* suggest that environments in which *Diuris* species live have been much more stable in the south-west than in the south-east.

The flowers of all species of *Diuris* that have yellow anthoxanthins appear to mimic pea flowers of genera within the tribes Mirbelieae and Bossiaeeae ('egg and bacon peas' – see Indsto *et al.* 2006) and this state is decisively reconstructed as the ancestral condition within the genus. According to the model of punctuated equilibria invoked above, early *Diuris* species could have evolved to fill a niche opportunity soon after the appearance of the Mirbeliae and Bossiaeeae more than 35 million years ago (Wojciechowski 2003), which can be considered a best estimate in the absence of molecular dating. The pea flowers of these groups have strongly conserved floral form, despite extensive evolutionary radiation. Consistent with the punctuated equilibria theory is the possibility that the pea and *Diuris* orchid flowers recognisable today may have existed in similar form for tens of millions of years. Recent evidence challenges the idea that orchids are recently evolved. The oldest fossil orchid (Ramirez *et al.* 2007), 15-20 million years old shows close resemblance in pollinaria structure to modern Goodyerinae. A fossil bee 65 – 70 million years old, now placed in the genus *Cretotrigona* has been noted to be remarkably modern in appearance and to be deeply nested within a eusocial clade (Michener and Grimaldi 1988; Engel 2000). Evidence for

the antiquity of bees has recently been extended even further to ~100 million years ago with the finding of a bee from early Cretaceous Burmese amber (Poinar and Danforth 2006). Perhaps one day a fossil Australian bee will be found, ancient yet modern in appearance, with an orchid pollinarium very like that of modern *Diuris* species attached to its face.

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Chapter 7

General Conclusions

1. Introduction

On the basis of the literature review and specific studies presented in this thesis, understanding of the pollination biology of the genus *Diuris* should be considered to be at an early stage. Previously published work on the genus (Beardsell *et al.* 1986), my work (this thesis, including Indsto *et al.* 2005, 2006, 2007) and my unpublished data (see Chapter 6) collectively suggest that guild mimicry is likely to be the pollination syndrome applicable to many *Diuris* species. I use the term pollination syndrome as a way to describe what seems a general pollination system, occurring in many species spread far and wide across the country. I suggest that the majority of *Diuris* species fall into some sort of syndrome based, either tightly or loosely, upon mimicry of the legumes commonly known as 'egg and bacon' peas. In this chapter I will avoid reiteration of what has been previously covered in this thesis, but rather I summarise the main findings, explore the implications of these results, and make some suggestions for future research.

2. General Pea Mimicry Appears Common in East Coast Species

In Chapter 3, I described the pollination of *Diuris maculata* in the Sydney region. The pollination system was very close to that found previously, about a thousand kilometres south at a site near Melbourne, by Beardsell *et al.* (1986). In the Melbourne population,

flowering of the orchid was synchronised with that of sympatric peas and a diversity of hymenopteran pollinators of both sexes was found. Although generally similar, there are some points of contrast to my findings for *Diuris maculata* in the Sydney region. A population of this orchid species at Scheyville National Park began flowering when very few other flowers were open. Interestingly, male *Trichocolletes venustus*, a pea specialist species and the only native bee observed in significant numbers, were also found at this time. Only an Ericaceous shrub, *Lissanthe strigosa* ssp. *strigosa* was flowering in significant numbers at this time and the bees had no option but to feed on this. By the time female *T. venustus* bees appeared, about 10 days later, many *Diuris maculata* flowers had been pollinated by the male bees and pea flowers were much more common. Presumably, these female bees may play a role in pollination later in the orchid flowering period, but this was not observed.

Anecdotal evidence suggests that numerous *Diuris* species and populations may have peak flowering either before, or in synchronisation with locally abundant 'egg and bacon' peas. Adaptation of timing of flowering is an important feature requiring more research. It is interesting to note, however, that there is no obvious difference in pollination outcome for *Diuris maculata* flowers that appeared earlier or in synchrony with sympatric peas. Rather, distinct differences in floral timing would appear to result in differences in the targeting of pollinators, whether primarily male bees, or mixed sex.

3. Strict Batesian Mimicry (close resemblance to a specific model) Appears Rare

I have observed previously that *Diuris* species range from being excellent mimics of pea flowers (e.g. *Diuris aequalis* and *Gompholobium huegelii*; Chapter 3, Figure 3.6), to being rather generic mimics (e.g. *Diuris maculata*), which shows what might be termed guild mimicry (a loose Batesian-type mimicry) of co-occurring rewarding model pea plants. Unlike common taxa, such as *D. maculata*, those that appear to be strict Batesian mimics are usually uncommon and restricted to a small number of populations.

4. Some *Diuris* Taxa Push the Boundaries of Mimicry – and Yet May Still Depend on Mimicry for Pollination

It is much easier to present a convincing case for close mimicry than loose mimicry. In *Diuris*, some species would appear to depend on pea mimicry and yet show only obscure visual resemblance to abundant model species. The example of *Diuris* sp. aff. *punctata* from Mellong Swamp in New South Wales provides an interesting dilemma (see Chapter 6, Preliminary pollination data for *Diuris* in 2003). This orchid has pea-like floral form, but a pink/purple base colour, unlike most common, sympatric, yellow-flowered 'egg and bacon' peas, but similar in colour (at least to human eyes) to less common peas such as *Mirbelia rubiifolia* and *Hardenbergia violacea*, which form common, but less dominant components of the pea flora where these orchids occur. This orchid occurs among abundant yellow-flowered *Dillwynia glaberrima* at Mellong Swamp, with similar scattered distribution as previously observed for *Diuris maculata* among similar peas. Interestingly,

the pollination outcome for this orchid was found to be indistinguishable to that expected for a yellow-flowered *Diuris* species when found in this spatial association with sympatric peas. It would appear that aspects of this orchids floral attributes (e.g. form of the flower) may be perceived by bees as similar to the sympatric peas, but clearly, colour cannot likely be perceived as similar (this could be proven using colorimetric analysis, but was not done in my research

I suggest that colour similarity cannot explain this behaviour and that strict Batesian mimicry does not apply. Instead, it would make more sense to hypothesise that the bees which respond to visual floral cues of yellow peas, nevertheless find this orchid attractive in the presence of yellow peas. This orchid would seem to be advertising visual cues of a more generic nature, in addition to pea-specific form-based cues, to which bees respond. This phenomenon could be explained as due to the 'magnet effect' (Peter and Johnson 2008), where the orchid shows features generally attractive to the bee floral visitors of the abundant pea flowers at this site. Dafni (1992) has developed concepts such as 'non-model mimicry' (see also Johnson 1994), in which mimicry is not targeted to a specific model. In a similar vein, it may be that this orchid displays some cues in an intensified manner relative to a model, such that it may be a 'super mimic', a concept introduced by Kullenberg (1961) in his studies of *Ophrys* pollination. A bee foraging on yellow legumes would in part be 'hard-wired' to seek these peas (see Laverty 1980 for discussion of bumblebee foraging behaviour), and also endowed with behavioural flexibility to be able to seek out the best floral rewards. The issue could be further complicated when one considers that some floral visitors may be pea specialists (such as

many *Trichocolletes* species) and others generalists, which show floral constancy to peas only when the floral rewards would warrant it. Perhaps some aspects of 'hard-wiring' of behaviour are fairly general in bees, and yet others may be specific to pea-specialist species. The idea that an orchid such as *D.* sp. aff. *punctata* from Mellong Swamp may be highly attractive to these bees, despite somewhat divergent floral features to an abundant model, is hard to understand and would be a suitable subject for future research.

As a final observation, phylogenetic data would suggest that species closely related to this orchid (*Diuris*, subg. *Diuris*, sect. *Purpureo-albae*) have evolved recently and rapidly to fill newly created environmental niches arising through the drying climate in eastern Australia. White base floral base colour with varying amounts of pink/purple suffusions represents a synapomorphy for this orchid group. The wide distribution and abundance of orchids with this colouration is suggestive that this divergence from ancestral colouration has met with evolutionary success.

5. Generalised Pollination in *Diuris* – Pollination Success Independent of Peas

In the case of *Diuris alba* at Munmorah, New South Wales (see Chapter 4), I presented evidence to show that it achieved high pollination success in the presence of sympatric *Dillwyna retorta.* This again may be due to the magnet effect (Peter and Johnson 2008). High pollination success was also achieved at a site in which peas were uncommon. In the case of this species, colorimetric analysis was used in combination with reference to

previous research into colour-based foraging errors of bees (Backhaus 1991; Dyer and Chittka 2004) to produce predictions which were compatible with research findings. This provided a theoretical model which could be compared with pollination outcomes and analysed to determine whether findings were compatible with the model. For contrast, the sympatric yellow-flowered (and putative pea mimic) *Diuris aurea* both in the presence and near absence of peas was studied. This latter orchid was found to have markedly higher reproductive success in the presence of peas than was found at the site where peas were rare. It was found that the methods of field pollination data collection resolved differences in the pollination outcome of these orchids in different environments, and that colorimetric analysis and theoretical considerations could predict these outcomes.

Phylogenetic analysis provided evidence that yellow base colour and pea mimicry are ancestral in *Diuris*. Therefore, it is proposed that *Diuris alba* shows divergence from ancestral pollination-related floral features. The high reproductive success of this orchid suggests that this divergence has met with selective advantage and may in part explain the evolutionary success of the group to it belongs.

The high pollination success of *Diuris alba* at a site where peas are uncommon may be due its flowers being generally attractive to bees, in combination with a meagre nectar reward. Interestingly, *Diuris alba* achieved higher pollination success than *D. aurea* in the presence of the abundant yellow, sympatric pea *Dillwynia retorta*. This can be in part explained by the presence of a meagre reward in *D. alba*, and its absence in *D. aurea*. Colorimetric analysis, including an ultraviolet component visible to hymenopteran insects,

including bees, shows that *D. aurea* is similar in colour to sympatric yellow peas, as expected for a mimic. On the other hand, *D. alba* is somewhat more distant in colour, but not so distant that colour-based foraging errors would be predicted to be uncommon. This prediction may seem odd to a human observer, but in the spectrally shifted hymenopteran visual system it would seem some residual colour similarity remains. Consideration of both floral form and colour in *D. alba* would suggest that features of floral mimicry of peas flowers have not been entirely lost and that some degree of pea floral mimicry may have a selective benefit to this species. Floral and pollination features of *Diuris alba* would seem to be highly adaptive to more diverse environments than would be possible with stricter floral pea mimicry.

6. The Basal Species *Diuris sulphurea*

This species is rather common and widespread in eastern Australia (Bishop 2000), with probably the widest distribution of *Diuris* species. It shows a preference for poorer eucalypt forests and heathland, and occurs in a tremendous variety of environments. In floral appearance it is pea-like, with considerable variation in size and features such brown markings. Although a probable floral mimic, the wide distribution is more suggestive of guild mimicry of yellow pea flowers than close Batesian mimicry, and in this sense it would seem comparable to a widespread species, such as *D. maculata*.

I have collected little pollination data for this species, but I have observed that it produces a small amount of nectar and shows high reproductive success in the general vicinity of pea flowers. On 12/10/2002 a bee (female *Lipotriches maurens*) in the vicinity of *D.* sp. aff. *punctata* was caught carrying an orchid pollinarium, that turned out from AFLP analysis to be from *D. sulphurea*. The bee was found to be carrying pollen of *Dillwynia glaberrima*. This bee is a medium-sized species and is a likely pollinator both of *D. sulphurea* and *D.* sp. aff. *punctata*. My only observation of *D. sulphurea* pollination outcomes was from a single colony at Mellong Swamp, New South Wales. It is interesting to re-examine what 'high reproductive success' means in this context. For an extended colony of growths (ramicauls), which might be considered an individual plant, only a small subset of the total growths may bear inflorescences. Even if those that do flower tend to bear significantly more fruits than would be the case for non-colony-forming species, in terms of fruit per plant biomass, or 'mature growth number', or other comparative measure, it is not clear whether the colony-forming *D. sulphurea* has overall higher reproductive success. This would require counts from a number of colonies to clearly resolve.

Phylogenetic analysis shows that this species is basal and sister to all other *Diuris* species (Indsto *et al.* 2009). It is therefore likely to show ancestral features of the genus. I propose that pea floral mimicry may be ancestral in *Diuris*. An interesting question to consider is the degree to which a species, such as *D. sulphurea* may show conserved ancestral features. A number of instances have been found of fossils of extinct bee species, millions of years old which show features startlingly like extant species (Ramirez et al.

2007; Michener and Grimaldi 1988). Features of *D. sulphurea*, such as its basal phylogenetic position and very wide distribution are compatible with a hypothesis that this species may have remained relatively unchanged for a long time. Certainly features such as generalised pea flower mimicry, nectar production and colony formation would be expected to contribute to long term survival.

7. Hypotheses of Evolutionary Drivers in *Diuris* Pollination and Ecological Niches

The low pollination success of floral mimics has been a challenge for pollination biologists to explain in terms of selective benefit. In the case of *Diuris*, it would appear that nectar production, putatively an ancestral feature of *Diuris* flowers has largely been lost (but apparently regained or retained in some species, such *D. alba*).

My research has concentrated on Australian east coast *Diuris* species and so may not be broadly applicable to the western Australian members of the genus. The majority of east coast species are not colony-forming, but rely on annual tuber replacement to survive. Death of individual plants must be frequent and these species therefore must depend on high levels of seedling recruitment to maintain populations. High fecundity would therefore seem to be advantageous for such species, unless low fecundity is linked to other strong benefits for these species.

Orchids are known to produce prodigious numbers of seeds, many of which must perish, so it is not an easy matter to estimate an appropriate level of seed production for an orchid, or any other plant for that matter. There is much evidence that the east coast of Australia has undergone significant drying over the last few million years (see Truswell and Harris 1982), which has been intensified in more recent times and is considered a major driver of extinctions of Australian megafauna (Wroe and Field 2006). A consequence of this is likely to have been the creation of much suitable habitat for *Diuris* orchids, with much habitat change generally. The ability of these orchids to adapt to environmental change and to colonise new habitats must have been linked with the capacity to generate many new forms with adaptive features. *Diuris* are known to mostly outcross (Beardsell *et al.* 1986) and taxa and populations show a great deal of variation. Floral mimicry and absence of rewards is favourable for outcrossing and the generation of genetic diversity (Johnson *et al.* 2000).

A high level of outcrossing may be beneficial, but evidently is not essential in all cases for maintenance of populations of a species. A number of orchid species e.g. *Orthoceras strictum* (see Bishop 2000) and *Cypripedium passerinum* (Catling and Bennett 2007) are self-pollinators and yet survive. This may be a common life history. The colony-forming and nectar-producing *Diuris sulphurea* is a highly successful species that probably has lower outcrossing than a non-rewarding species such as *D. maculata*. However, *D. sulphurea* appears to be a single taxon, whereas *D. maculata* might be termed a 'super species' (especially if close relatives such as *D. goonooensis*, *D. platichila* and *D. pardina*

are sunk into this latter taxon), either of one highly variable species, or a number of closely related species undergoing rapid, radiative evolution.

Diuris aequalis is proposed to be a Batesian mimic of *Gompholobium* spp. peas. This species is relatively uncommon. Batesian mimicry might be expected to evolve from more generalised mimicry of this general type under conditions of habitat and/or model isolation. Perhaps the Batesian mimic is at risk of becoming an evolutionary dead-end, especially if the circumstances to which it is exquisitely adapted change to its detriment.

Diuris alba has been proposed to have more generalised pollination than related species, which are proposed to depend on pea mimicry for pollination. This plant has been found frequently to occur in more diverse habitats than putative pea mimics. Interestingly, pollination data show that this species has not lost the capacity for pollination in the presence of peas, but has been able to extend its habitat range by evolving more generalised pollination features. It is an interesting question whether *D. alba* would have similar pollination success to a yellow-flowered *Diuris* species in the presence of yellow-flowered peas without nectar production – would the 'magnet effect' be enough to ensure adequate pollination? It might be expected that other species closely related to *D. alba* may also have evolved generalised pollination and *D. arenaria* is a likely example. Other less closely related *Diuris* species may have similarly evolved generalised pollination mechanisms.

These observations and hypotheses for eastern Australian *Diuris* species are likely to apply to a significant degree to western Australian species. However, phylogenetic analysis clearly shows greater genetic variation in western Australian species of *Diuris* subg. *Hesperodiuris* than has been found for eastern species. It is likely that this will be reflected in similarly high diversity in pollination mechanisms that will be in some respects distinct to that observed in this study.

8. A Consideration of Experimental Methods

In my field work I have used what can be described as 'natural experiments' i.e. I have endeavoured to find in natural populations of orchids features of the plants spatial distribution, timing of flowering and other biotic factors, opportunities which lend themselves to answer questions about their biology. I have not undertaken studies to test the outcome of experimental manipulation in the field. Certainly some classic methods, such as the 'interview technique' used by Johnson et al. (2003b), where an inflorescence of an orchid is presented in the flight path of an insect foraging on a model plant species, so it can choose between this and other plants of the model species would have been impractical in my field work. In particular, it would have been difficult with the fast-flying bee pollinators of species such as *Diuris maculata*. I often saw these insects for only fractions of a second – by the time I registered their presence they were usually gone. However, manipulation of inflorescence features has also been used by Johnson *et al.* (2003a). In this case the cymose floral presentation of a successful orchid mimic was manipulated into an artificial raceme, with reduced pollination success. Transplant experiments may also help

get to the bottom of the relationship of spatial distribution of orchid mimics relative to model species, particularly with regard to the observation that clustered orchids some distance form a group of model species enjoy the highest level of reproductive success.

In my work I was not averse to such approaches, but couldn't think of good experiments applicable to *Diuris* orchids in the field. Further experimental studies into the role of colour (and perhaps its relationship to form) in pollination outcome of *Diuris* species is clearly warranted. A better understanding of the relationship of orchid colour, form, and fragrance in pollination outcome may depend on better understanding of bee sensory perception in terms of all these aspects.

The collection of basic pollination data is still of great importance. So little has been collected that a basic understanding of *Diuris* pollination is at an early stage. I consider that *Diuris* orchid species offer tremendous opportunities for those interested in mimicry - the complex interactions of a highly variable plant group, the many (presumably mostly hymenopterous) pollinators and the habitats in which they occur will, I expect, defy a simple understanding.

9. DNA-based identification of Orchid Pollinaria

Considerable effort was undertaken to develop an AFLP method for the identification of *Diuris* species from various plant tissues, including orchid pollinaria. It was soon realised

that this would be of great value for situations where more than *Diuris* species was sympatric and had overlapping flowering period. This was found to be useful in two pollination studies: the sympatric species *Diuris alba* and *D. aurea* at Munmorah; and *D.* sp. aff. *punctata* and *D. sulphurea* at Mellong Swamp. In both cases the ability to identify the species source of orchid pollinaria was critical to understanding pollinator behaviour at these sites. It was later found that sequence analysis of the ITS regions could also successfully distinguish these species.

10. Mapping of Pollination Systems on to Phylogenetic Trees

The efficacy of this approach was most convincingly made by Johnson *et al.* (1998) in a study of the phylogeny and radiation of pollination systems in *Disa* (Orchidaceae). Figure 4 in their study is quite stunning in its illustration of the complexity of pollination systems in the genus and how, in different branches of the tree, pollination by various insect groups has independently arisen. Phylogenetic analysis has great value in revealing homoplasy in pollination mechanisms, especially when it is considered that extensive morphological homoplasy may obscure species relationships.

Diuris can hardly be expected to show this level of diversity in pollination systems, but the techniques of mapping pollination systems on to a phylogenetic tree can be expected to help focus efforts in understanding pollination systems in this orchid group. Some progress has been made in this regard in this project – a robust phylogeny for *Diuris* has been completed (see Chapter 5) and it shows the evolutionary relationships of a recent

classification scheme (Jones 2000; Jones and Clements 2006). In time, it is to be hoped that this tree may be populated by many examples of *Diuri*s pollination variants.

Phylogenetic analysis has powerfully contributed to pollination studies in *Diuris* by elucidating species relationships. Knowledge of whether species are closely, or more distantly related, or possibly similar in appearance due to homoplasy and shared pollination features is of great help in focusing studies. As an example, it is now clear that a detailed study of the pollination biology of the basal *Diuris* species *D. sulphurea* can be expected to contribute much to understanding *Diuris* pollination more generally.

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Appendices

Appendix 1

Insect collection details for 2001, 2002 and 2003.

Table A1.1. Details of insects captured during field research in 2001. Identifications by Dr Michael Batley. Place names are abbreviated: Scheyville NP = Scheyville National Park in western Sydney, NSW. LMSRA = Lake Macquarie State Recreation Area, Munmorah, NSW. Mellong Swamp is located approximately 110 km north of Windsor on the Putty Road, NSW.

Bee #	Date	Particulars	Location	Sex
1	13/8/2001	By *Daviesia/Hardenbergia* ~1.00pm	Scheyville NP	M
2	13/8/2001	As above by *Hardendergia*	"	M
3	13/8/2001	Small bee on *Daviesia* ~ 1.00 pm	"	
4	15/8/2001	Male bee on *Daviesia/Hardenbergia* ~ 12.40 pm	"	M
5	15/8/2001	On *Hardenbergia/Daviesia*	"	F
6	21/8/2001	Honeybee worker on *Daviesia* (not much pollen on legs) 10.30 am	"	F
7	21/8/2001	" " " " ~10.40 am	"	F
8	21/8/2001	" " " " ~ 10.50 am - conspicuous pollen masses on legs	"	F
9	24/8/2001	On *Hardenbergia*	"	M
10	24/8/2001	Female *Trichocolletes*? On *Daviesia* ~ 12.45 pm	"	F
11	25/8/2001	~ 11.20 am On *Hardenbergia* **with orchid pollinarium on head** (only 1 of 8 caught)	"	M

12	25/8/2001	On *Hardenbergia* ~ 12.45 pm	"	F
13	25/8/2001	Near *Hardenbergia* ~ 12.00 pm	"	F
14	25/8/2001	Caught on *Hardenbergia* ~ 12.20 pm	"	F
15	25/8/2001	Caught near *Hardenbergia* ~ 12.30 pm	"	F
16	1/9/2001	Native bee on *Dillwynia retorta*	LMSRA	F
17	1/9/2001	Honeybee worker on *Pimelea linifolia* ssp. *linifolia*	"	F
18	1/9/2001	" " " "	"	F
19	1/9/2001	Large fly on *Pimelea linifolia* ssp. *linifolia*- general interest	"	
20	1/9/2001	Another fly on *Pimelea linfolia* ssp. *lnifolia*	"	
21	1/9/2001	Native bee on *Dillwynia retorta*	"	F
22	1/9/2001	Native bee on *Pimelea linifolia* ssp. *linifolia*	"	F
23	1/9/2001	Wasp seen visiting both *Pimelea* and *Dillwynia*	"	
24	1/9/2001	Native bee on *Dillwynia retorta*	"	M
25	1/9/2001	Honeybee on *Dillwynia retorta*	"	F
26	8/9/2001	Halictid bee? On *Xanthorrhea* sp.	"	F
27	8/9/2001	Honeybee **with orchid pollinarium**	"	F
28	8/9/2001	Native bee on *Dillwynia retorta*	"	F
29	15/9/2001	Bee seen visiting visiting *Diuris aurea* - resting on nearby grass 12.15 pm	"	F
30	15/9/2001	Small butterfly which landed on *D. aurea* and probed before resting nearby	"	

31	15/9/2001	Crane fly common in area - but an unlikely pollinator	"	
32	15/9/2001	Pollinarium removed from *D. aurea*	"	
33	19/9/2001	Syrphid fly on *Dillwynia retorta* - warm sunny conditions ~ 10.30 am	"	
34	19/9/2001	Native bee caught visiting *D. aurea* ~ 11.00 am	"	F
35	19/9/2001	Native bee caught visiting *Dillwynia retorta*	"	F
36	19/9/2001	Native bee caught on *Dillwynia* **with apparent orchid pollinarium**	LMSRA	F
37	19/9/2001	Fly found resting on *D. aurea*	"	
38	19/9/2001	*Diuris aurea* pollinarium	"	
39	28/9/2001	Large wasp found visiting *Burchardia umbellata* - a possible pollinator of *D. alba*?	"	
40	28/9/2001	Beetle resting on *D. aurea* lateral petals	"	
41	28/9/2001	Butterfly seen visiting *D. aurea*	"	
42	29/9/2001	*Dillwynia tenuifolia* now comonly in flower - some native bee visitors - one caught	Scheyville NP	F
43		No sample	"	
44		No sample	"	
45	12/10/2001	Orange native bee caught on *Dillwynia glaberrima* ~ 10.45 am	Mellong Swamp	M
46	12/10/2001	Female of above species? Also visiting *Dillwynia glaberrima*	"	F
47	12/10/2001	Another bee species - also visiting *Dillwynia glaberrima*	"	F
48	12/10/2001	Same species - near *Diuris* sp. aff. *punctata* - **with orchid pollinarium on face**	"	F

49	12/10/2001	Honeybee **with orchid pollinarium on face**	"	F
50	12/10/2001	*Diuris* sp. aff. *punctata* pollinarium	"	
51	13/10/2001	Native bee - seen hovering by *Dillwynia glaberrima* ~ 10.20 am	"	M
52	13/10/2001	Small native bee on *Dillwynia glaberrima* ~ 10.35 am	"	M
53	13/10/2001	Small native bee on *Dillwynia glaberrima*. Similar bee seen near D. sulphurea	"	F
54	13/10/2001	Small native bee on *Dillwynia glaberrima* ~ 12.30 pm	"	F
55	20/10/2001	Small bee on *Dillwynia glaberrima* ~ 11.10 am	"	F
56	29/11/2001	Bee on large highly dissected yellow daisy ~ 11.30 am	Kanangra - BRCG	F
57	29/11/2001	A small bee also on this daisy	"	M
58	29/11/2001	Samples 58-65: Small bees on *Pultenaea polifolia* - Cattle Impoundment, Kanangra Boyd	Kanangra Boyd NP	F
59	"		"	M
60	"		"	M
61	"		"	F
62	"		"	F
63	"		"	M
64	"		"	F
65	"		"	F
66	29/11/2001	*Diuris chryseopsis* pollinaria	"	
67	29/11/2001	Bee caught on *D. chryseopsis* - **with orchid pollinarium remains**	"	F

68	29/11/2001	Sames bee species? – caught on nearby yellow daisy	"	F

Table A1.1 (Cont'd)

Bee #	Genus	Subgenus	Species	Pollen Analysis	AFLP of Orchid Pollen
1	*Trichocolletes*	*Trichocolletes*	*venustus*	*Daviesia/Hardenbergia* 93:7	
2	*Trichocolletes*	*Trichocolletes*	*venustus*	*Daviesia/Hardenbergia* 43:1	
3	Wasp			No pollen	
4	*Trichocolletes*	*Trichocolletes*	*venustus*	No pollen	
5	*Trichocolletes*	*Trichocolletes*	*venustus*	142 *Daviesia,* No *Hardenbergia* +1 unknown	
6	*Apis*		*mellifera*	124 *Daviesia,* No *Hardenbergia*	
7	*Apis*		*mellifera*	Hundreds of , No other	
8	*Apis*		*mellifera*	178 *Daviesia*, No other	
9	*Trichocolletes*	*Trichocolletes*	*venustus*	No pollen	
10	*Trichocolletes*	*Trichocolletes*	*venustus*	No pollen	
11	*Trichocolletes*	*Trichocolletes*	*venustus*	No pollen	Matches *D. maculata*
12	*Trichocolletes*	*Trichocolletes*	*venustus*	No pollen	
13	*Trichocolletes*	*Trichocolletes*	*venustus*	*Hardenbergia/Daviesia* 52:7	
14	*Trichocolletes*	*Trichocolletes*	*venustus*	*Hardenbergia/Daviesia* 19:17	
15	*Trichocolletes*	*Trichocolletes*	*venustus*	No pollen	
16	*Leioproctus*	*Euryglossidia*	*rectangulatus*	Some clumps of *Dillwynia* pollen	
17	*Apis*		*mellifera*	One *Pimelea* grain	
18	*Apis*		*mellifera*	One *Pimelea* grain	

19	No specimen				
20	Fly				
21	*Leioproctus*	*Euryglossidia*	*rectangulatus*	Lots of *Dillwynia* grains	
22	*Exoneura*	*Exoneura*		A few grains of *Dillwynia* and *Pimelea*	
23	No specimen				
24	*Leioproctus*	*Euryglossidia*	*rectangulatus*	Lots of *Dillwynia* grains only	
25	*Apis*		*mellifera*	mostly *Dillwynia* grains	
26	*Amphylaeus*	*Amphylaeus*	*morosus*	Some *Dillwynia* pollen + *Xanthorrhea*?	
27	*Apis*		*mellifera*	*Dillwynia* plus some orchid pollen	Matches *D. aurea*
28	*Leioproctus*	*Euryglossidia*	*rectangulatus*	A few grains of *Dillwynia* only	
29	*Megachile*		*leucopyga*	Lots of *Dillwynia* pollen only	
30	No specimen				
31	No specimen				
32	No specimen				
33	No specimen				
34	*Exoneura*	*Exoneura*		*Dillwynia* pollen only	
35	*Exoneura*	*Exoneura*		No pollen	
36	*Exoneura*	*Exoneura*		*Dillwynia* pollen plus something else - not orchid	No result
37	No specimen				
38	No specimen				
39	No specimen				
40	No specimen				
41	No specimen				

42	*Mellitosmithia*		*subtilis*	*Dillwynia* pollen	
43	No specimen				
44	No specimen				
45	*Trichocolletes*	*Trichocolletes*	*marginatulus*	No pollen	
46	*Trichocolletes*	*Trichocolletes*	*marginatulus*	*Dillwynia* grains + unknown	
47	*Lipotriches*	*Paulynomia*	*moerens*	Lots of *Dillwynia* pollen only	
48	*Lipotriches*	*Paulynomia*	*moerens*	Some *Dillwynia* pollen, mostly orchid pollen	Matches *D. sulphurea*
49	*Apis*		*mellifera*	*Dillwynia* pollen + other?	Matches *D.* sp. aff. *punctata*
50	No specimen				
51	*Trichocolletes*	*Trichocolletes*	*marginatulus*	*Dillwynia* pollen	
52	*Megachile*		near *paracallida*	No pollen	
53	*Exoneura*	*Brevineura*		*Dillwynia* pollen	
54	*Lasioglossum*	*Chilalictus*	*pulvivectum*	*Dillwynia* pollen	
55	*Euhesma*			No pollen	
56	*Lasioglossum*	*Parasphecodes*	*lacthium*	One daisy grain seen	
57	*Lasioglossum*	*Chilalictus*	*clelandi*	No pollen	
58	*Lasioglossum*	*Chilalictus*	*clelandi*	Moderate amounts *Pultenaea* pollen	
59	*Lasioglossum*	*Chilalictus*	*clelandi*	No pollen	
60	*Lasioglossum*	*Chilalictus*	*clelandi*	Moderate amounts *Pultenaea* pollen	
61	*Lasioglossum*	*Chilalictus*	*clelandi*	Moderate amounts *Pultenaea* pollen	
62	*Lasioglossum*	*Chilalictus*	*sculpturatum*	No pollen	
63	*Lasioglossum*	*Chilalictus*	*clelandi*	*Pultenaea* pollen	
64	*Lasioglossum*	*Parasphecodes*	*melbournense*	Lots of *Pultenaea* pollen + larger legume pollen	

65	*Lasioglossum*	*Chilalictus*	*clelandi*	*Pultenaea* pollen	
66	No specimen				
67	*Lasioglossum*	*Chilalictus*	*clelandi*	Mostly *Pultenaea* pollen + some orchid pollen	Matches "maculata" type
68	*Lasioglossum*	*Chilalictus*	*clelandi*	*Pultenaea* pollen + one daisy grain	

Table A1.2. Details of insects caught during field research in 2002. Bee identifications by Dr Michael Batley. Location details not given above are: Lake Parramatta; Lake Parramatta Reserve, Parramatta, NSW; Munmorah SRA = Munmorah State Recreation Area, Munmorah, NSW; Tomaree NP = Tomaree Natinal Park, NSW.

Bee #	Date	Location	Particulars	Genus	Subgenus	Species
1	30/08/2002	Scheyvillle NP		*Trichocolletes*	*Trichocolletes*	*venustus*
2	"	"		*Trichocolletes*	*Trichocolletes*	*venustus*
3	"	"		*Trichocolletes*	*Trichocolletes*	*venustus*
4	"	"	on *Hardenbergia*	*Trichocolletes*	*Trichocolletes*	*venustus*?
5	02/09/2002	Lake Parramatta	on *Hardenbergia*	*Trigona*	*Heterotrigona*	*carbonaria*
6	02/09/2002	"	on *Hardenbergia*	*Lipotriches*	*Austronomia*	*australica*
7	02/09/2002	"		*Megachile*		*ferox*
8	07/09/2002	Scheyville NP	on *Hardenbergia*	*Trichocolletes*	*Trichocolletes*	*venustus?*
9	08/09/2002	"	by *Hardenbergia*	*Trichocolletes*	*Trichocolletes*	*venustus*
10	"	"	by *Hardenbergia*	*Trichocolletes*	*Trichocolletes*	*venustus*
11	"	"		*Apis*		*mellifera*
12	"	"	on *Daviesia*	*Hylaeus*	*Planihylaeus*	*trilobatus*
13	15/09/2002	LMSRA	on *Dillwynia retorta*	*Exoneura*	*Exoneura*	sp.

14	"	"	on *Dillwynia retorta*	*Exoneura*	*Exoneura*	sp.
15	"	"	on *Dillwynia retorta*	*Exoneura*	*Exoneura*	sp.
16		Munmorah SRA	on *Grevillea sericea*	*Apis*		*mellifera*
20	"	Munmorah SRA	on *Isopogon* sp.	*Leioproctus*	*Leioproctus*	sp.
23	19/09/2002	Tomaree NP	on *Dillwynia* sp.	*Lasioglossum*	*Parasphecodes*	*carbonarium*
24	20/09/2002	Tomaree NP		*Leioproctus*		*tuberculatus*
25	20/09/2002	Tomaree NP	on *Hardenbergia*	*Trichocolletes*	*Trichocolletes*	*venustus*
26	26/09/2002	Tomaree NP	on *Hardenbergia*	*Lipotriches*	*Austronomia*	M2"
27	26/09/2002	Tomaree NP	on *Hardenbergia*	*Lipotriches*	*Austronomia*	M2"
28	26/09/2002	Tomaree NP	on Hardenbergia	*Lipotriches*	*Austronomia*	M2"
29	26/09/2002	Tomaree NP	on *Bossiaea ensata*	*Lasioglossum*	*Parasphecodes*	*clarigaster*
30	26/09/2002	Tomaree NP	on *Hardenbergia*	*Lipotriches*	*Austronomia*	M2"
31	18/10/2002	Mellong Swamp	on *Dillwynia glaberrima*	*Exoneura*	*Exoneura*	sp.
32	18/10/2002	Mellong Swamp	on *Dillwynia glaberrima*	*Megachile*		*heliophila*
33	18/10/2002	Mellong Swamp	on *Dillwynia glaberrima*	*Exoneura*	*Exoneura*	sp.
34,35,36	18/10/2002	Mellong Swamp	on *Dillwynia glaberrima*	*Exoneura*	*Exoneura*	sp.
37	18/10/2002	Mellong Swamp	on *Dillwynia glaberrima*	*Trichocolletes*	*Trichocolletes*	*marginatulus*
38	18/10/2002	Mellong Swamp	on *Dillwynia glaberrima*	*Trichocolletes*	*Trichocolletes*	*marginatulus*
39	18/10/2002	Mellong Swamp	on *Dillwynia glaberrima*	*Lasioglossum*	*Chilalictus*	*orbatum*
40	18/10/2002	Mellong Swamp	on *Dillwynia glaberrima*	*Trigona*	*Heterotrigona*	*carbonaria*

Table A1.2 (Cont'd)

Bee #	Sex	Pollen Analysis	Comments (bee ID)
1	M	*Hardenbergia* (4) *Daviesia* (11)	genitalia checked
2	M	*Hardenbergia* (3) *Daviesia* (41)	genitalia checked
3	M	*Hardenbergia* (28) *Daviesia* (2)	genitalia checked
4	F	*Hardenbergia* (45) *Daviesia* (2)	chars consistent with *T. venustus*
5	F worker		
6	F		
7	M		
8	F		chars consistent with *T. venustus*
9	M		genitalia checked
10	M		genitalia checked
11			
12	M		
13	F	Lots of *Dillwynia retorta* pollen	
14	F	Lots of *Dillwynia retorta* pollen (39)	
15	F	Lots of *Dillwynia retorta* (29) + (3) v sm grains (*Mirbelia*?)	
16		*Dillwynia retorta* (15) + *Grevillea sericea* (2)	
20	F	Large triangular pollen (no ref., but typical of Proteaceae)	from literature description, possibly *L. helichrysi*
23	F	Lots of *Pultenaea* sp. (sm. round) + some *Dillwynia retorta*	
24	F	Lots of *Pultenaea* sp. + some *Dillwynia* or *Hardenbergia*	subgenus is technically *Lamprocolletes*
25	M	Lots of *Hardenbergia* pollen	genitalia checked
26	F	Mostly *Hardenbergia* + *Pultenaea* (~10:1)	probably *L. submoerens*
27	F	Mostly *Hardenbergia* + *Pultenaea* (~10:1)	probably *L. submoerens*

28	F	Mostly *Hardenbergia* + *Pultenaea* (~10:1)	probably *L. submoerens*
29	F	Lots of *Pultenaea* sp. (sm. round)	
30	F	Mostly *Hardenbergia* + some *Pultenaea*	probably *L. submoerens*
31	F	Mostly *Dillwynia glaberrima* + some unidentified	
32	F	*Dillwynia glaberrima* (1 grain!)	probably *L. submoerens*
33	F		
34,35,36	F	a few *Dillwynia* grains only	
37	M	Lots of small unidentified pollen + some *Dillwynia*	
38	F	Mostly *Dillwynia glaberrima* + some unidentified	
39	F	Mostly *Dillwynia glaberrima* + some unidentified	widespread and common
40	F worker	No pollen	

Table A1.2 (Cont'd)

Bee #	Comments (field)
1	pollinarium remnants match *D. maculata* (AFLP)
2	pollinarium remnants match *D. maculata* (AFLP)
3	pollinarium remnants match *D. maculata* (AFLP)
4	
5	not *Diuris* site
6	yellow stripes on abdomen
7	
8	

9	Pollinarium remnants?
10	with orchid pollinarium
11	
12	small native bee
13	
14	
15	
16	
20	
23	
24	
25	
26	
27	same as above?
28	none of three with pollinarium
29	very small
30	
31	common on site
32	
33	small native bee
34,35,36	3 small bees caught at once
37	no sign of orchid pollen
38	
39	a new native bee?

40	small bee

Table A1.3. Details of insects caught during field research in 2003. Bee identifications by Dr Michael Batley. Location details not given above : Castlereagh NR = Castlereagh Nature Reserve, near Windsor, NSW.

Bee #	Date	Location	Particulars	Family	Genus	Subgenus	Species
1	08/08/2003	Munmorah SRA	on *Hardenbergia*		*Trichocolletes*	*Trichocolletes*	*venustus*
2	08/08/2003	Munmorah SRA	on *Hardenbergia*		*Apis*		*mellifera*
3	08/08/2003	Munmorah SRA	on *Hardenbergia*		*Trichocolletes*	*Trichocolletes*	*venustus*
4	08/08/2003	Munmorah SRA	on *Diuris praecox*		*Lasioglossum*	*Chilalictus*	*orbatum*
8	27/08/2003	LMSRA	on *Dillwynia retorta*		*Lasioglossum*	*Chilalictus*	*orbatum*
9	27/08/2003	LMSRA	on *Dillwynia retorta*		*Leioproctus*	*Euryglossidia*	*rectangulatus*
10	27/08/2003	Munmorah SRA	on *Pultenaea* sp.		*Lasioglossum*	*Chilalictus*	*orbatum*
11	27/08/2003	Munmorah SRA	on *Hardenbergia*		*Lasioglossum*	*Chilalictus*	*orbatum*
12	27/08/2003	Munmorah SRA	near *Hardenbergia*		*Trichocolletes*	*Trichocolletes*	*venustus*
13	27/08/2003	Munmorah SRA	on *Grevillea sericea*		*Apis*		*mellifera*
14	27/08/2003	Munmorah SRA	on *Hardenbergia*		*Lasioglossum*	*Chilalictus*	*orbatum*
15	05/09/2003	Munmorah SRA	on *Diuris praecox*		*Braunsapis*		*unicolor*
17	05/09/2003	Munmorah SRA (rocks)	on *Diuris alba*		*Exoneura*	*Exoneura*	sp.
18	05/09/2003	Munmorah SRA (rocks)	on *Diuris alba*		*Exoneura*	*Exoneura*	sp.
19	05/09/2003	Munmorah SRA (rocks)	on *Diuris alba*		*Exoneura*	*Exoneura*	sp.
20	05/09/2003	Munmorah SRA (rocks)	on *Diuris alba*		*Exoneura*	*Exoneura*	sp.

21	10/09/2003	Munmorah SRA (rocks)	on *Diuris alba*		*Exoneura*	*Exoneura*	sp.
22	10/09/2003	Munmorah SRA (rocks)	on *Diuris alba*		*Exoneura*	*Exoneura*	sp.
23	10/09/2003	Munmorah SRA (rocks)	on *Diuris alba*	*Euryinae* (sawfly)	*Eurys*		*pulcher*
24	10/09/2003	Munmorah SRA (rocks)	on *Diuris alba*		*Exoneura*	*Exoneura*	sp.
26	10/09/2003	Munmorah SRA (rocks)	on *Diuris alba*		*Paralastor*		sp.
27	10/09/2003	Munmorah SRA (rocks)	on *Diuris alba*		*Exoneura*	*Exoneura*	sp.
28	11/09/2003	Tomaree NP	on *Pultenaea* sp.		*Leioproctus*	*Leioproctus*	*platycephalus*
29	11/09/2003	Tomaree NP	on *Pultenaea* sp.		*Leioproctus*	*Leioproctus*	*platycephalus*
30	11/09/2002	Tomaree NP	on *Pultenaea* sp.		*Apis*		*mellifera*
31	11/09/2003	Tomaree NP	on *Pultenaea* sp.		*Exoneura*	*Exoneura*	sp.
32	11/09/2003	Tomaree NP	on *Pultenaea* sp.		*Apis*		*mellifera*
33	11/09/2003	Tomaree NP	on *Pultenaea* sp.		*Exoneura*	*Exoneura*	sp.
34	11/09/2003	Tomaree NP	on *Pultenaea* sp.		*Exoneura*	*Exoneura*	sp.
35	11/09/2003	Tomaree NP			*Exoneura*	*Exoneura*	sp.
36	11/09/2003	Tomaree NP	on *Dillwynia retorta*		*Exoneura*	*Exoneura*	sp.
37	11/09/2003	Tomaree NP	on *Hardenbergia*		*Trichocolletes*	*Trichocolletes*	*venustus*
38	11/09/2003	Tomaree NP	on *Hardenbergia*		*Trichocolletes*	*Trichocolletes*	*venustus*
39	11/09/2003	Tomaree NP	on *Pultenaea* sp.		*Leioproctus*	*Leioproctus*	*platycephalus*
40	11/09/2003	Tomaree NP	on *Pultenaea* sp.		*Exoneura*	*Exoneura*	sp.
41	12/09/2003	Tomaree NP	on *Pultenaea* sp.		*Exoneura*	*Exoneura*	sp.
42	12/09/2003	Tomaree NP	on *Dillwynia retorta*		*Exoneura*	*Exoneura*	sp.
43	12/09/2003	Tomaree NP	on *Pultenaea* sp.		*Apis*		*mellifera*
44	19/09/2003	LMSRA	on *Dillwynia retorta*		*Exoneura*	*Exoneura*	sp.
45	19/09/2003	Munmorah SRA	on *Diuris alba*		*Apis*		*mellifera*

46	23/09/2003	Castlereagh NR	on *Bossiaea obcordata*		*Apis*		*mellifera*
47	23/09/2003	Castlereagh NR	on *Bossiaea obcordata*		*Megachile*		*apicata*
48	23/09/2003	Castlereagh NR	on *Bossiaea obcordata*		*Lasioglossum*	*Chilalictus*	*chapmani*
49	23/09/2003	Castlereagh NR	on *Bossiaea obcordata*		*Lasioglossum*	*Chilalictus*	*convexum*
50	25/09/2003	Castlereagh NR	on *Bossiaea obcordata*		*Lipotriches*	*Austronomia*	*moerens*
51	25/09/2003	Castlereagh NR	on *Bossiaea obcordata*		*Lasioglossum*	*Chilalictus*	*chapmani*
52	25/09/2003	Castlereagh NR	on *Bossiaea obcordata*		*Megachile*		sp.
53	25/09/2003	Castlereagh NR	on *Bossiaea obcordata*		*Lipotriches*	*Austronomia*	*excellens*
54	25/09/2003	Castlereagh NR	on *Diuris aurea*		*Exoneura*	*Exoneura*	sp.
55	08/10/2003	Castlereagh NR	on *Stylidium*/*Pultenaea* sp.		*Megachile*		sp.
56	08/10/2003	Castlereagh NR	on *Stylidium*		*Lipotriches*	*Austronomia*	*moerens*
57	08/10/2003	Castlereagh NR	on *Pultenaea* sp.		*Megachile*		sp.
58	08/10/2003	Castlereagh NR	on *Pultenaea* sp.		*Megachile*		sp.
59	08/10/2003	Castlereagh NR	on *Xanthorrhea* sp.		*Lasioglossum*	*Chilalictus*	*erythrurum*
60	08/10/2003	Castlereagh NR	on *Pultenaea* sp.		*Lipotriches*	*Austronomia*	*excellens*
61	08/10/2003	Castlereagh NR	on *Diuris aurea*		*Lasioglossum*	*Chilalictus*	*erythrurum*
62	08/10/2003	Castlereagh NR	on *Pultenaea* sp.		*Lipotriches*	*Austronomia*	*excellens*

63	08/10/2003	Castlereagh NR	on *Stylidium*		*Lipotriches*	*Austronomia*	*excellens*
64	08/10/2003	Castlereagh NR	on *Pultenaea* sp.		*Megachile*		sp.
66	11/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
67	11/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
69	11/10/2003	Mellong Swamp	approaching *Dillwynia glaberrima*		*Lipotriches*	*Austronomia*	M2"
70	11/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
71	11/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
73	15/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Euhesma*		sp.
74	15/10/2003	Mellong Swamp	on *Diuris* sp. aff. *punctata*		*Trigona*	*Heterotrigona*	*carbonaria*
75	15/10/2003	Mellong Swamp	on *Diuris* sp. aff. *punctata*		*Trigona*	*Heterotrigona*	*carbonaria*
76	15/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
77	15/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Lasioglossum*	*Chilalictus*	*victoriellum*
78	15/10/2003	Mellong Swamp	approaching *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
79	15/10/2003	Mellong Swamp	on *Diuris* sp. aff. *punctata*		*Exoneurella*		*lawsoni*
80	15/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Euhesma*		sp.

81	15/10/2003	Mellong Swamp	on *Diuris* sp. aff. *punctata*		*Trigona*	*Heterotrigona*	*carbonaria*
82	15/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
83	15/10/2003	Mellong Swamp	on *Diuris* sp. aff. *punctata*		*Trigona*	*Heterotrigona*	*carbonaria*
84	15/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Exoneura*	*Exoneura*	sp.
85	15/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Megachile*	*Austrochile*	*tasmanica*
86	15/10/2003	Mellong Swamp	on *Diuris* sp. aff. *punctata*		*Exoneura*	*Exoneura*	sp.
87	15/10/2003	Mellong Swamp	on *Diuris* sp. aff. *punctata*		*Trigona*	*Heterotrigona*	*carbonaria*
88	15/10/2003	Mellong Swamp	on *Diuris* sp. aff. *punctata*		*Trigona*	*Heterotrigona*	*carbonaria*
89	15/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
90	21/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
91	21/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
92	21/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	*marginatulus*
93	21/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Hylaeus*	*Planihylaeus*	near *probligenatus*
94	21/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Trichocolletes*	*Trichocolletes*	i
95	21/10/2003	Mellong Swamp	on *Dillwynia*		*Exoneura*	*Exoneura*	sp.

			glaberrima				
96	21/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Lasioglossum*	*Chilalictus*	*chapmani*
97	21/10/2003	Mellong Swamp	on *Dillwynia glaberrima*		*Megachile*		*tasmanica*

Table A1.3 (Cont'd)

Bee #	**Sex**	**Pollen Analysis**
1	F	Mostly *Hardenbergia* plus some *Pultenaea*
2		Little pollen - mixed legume
3	M	*Hardenbergia* (~20 grains)
4	F	Some orchid pollen
8	F	A few *Dillwynia* grains
9	F	A few *Dillwynia* grains
10	F	A few *Pultenaea* grains
11	F	*Hardenbergia* plus a few *Philotheca*, plus *Comesperma*
12	F	Lots of *Hardenbergia* plus a few other
13		Mostly *Burchardia*
14	F	No pollen
15	F	
17	F	Mostly *Dillwynia* or *Hardenbergia* pollen
18	F	No pollen
19	F	Mostly *Pultenaea* + some orchid pollen
20	F	1 legume grain

21	F	No pollen
22	F	A few orchid pollen grains plus *Comesperma*
23		
24	F	2 orchid pollen grains
26		No pollen
27	F	No pollen
28	F	almost all *Pultenaea* pollen
29	F	Lots of *Bossiaea heterophylla* pollen
30		Masses of *Pultenaea* pollen plus some *Dillwynia*
31	F	Lots of *Pultenaea* pollen
32		Lots of *Pultenaea* pollen
33	F	Lots of *Pultenaea* pollen
34	F	1 *Pultenaea* grain
35	F	No pollen
36	F	*Dillwynia* + *Baeckea ramosissima* + a few others
37	F	~1:1 *Hardenbergia*: *Pultenaea* + a few other
38	F	Mostly *Hardenbergia* plus a few other
39	F	Mostly *Pultenae*a + a few other
40	F	A few *Pultenaea* grains
41	F	Mostly *Pultenaea*
42	F	Mostly *Dillwynia* plus a few other
43		Masses of *Pultenaea* pollen only
44	F	Lots of *Dillwynia* pollen + some *Bossiaea* + a few *Mirbelia*?
45		No pollen

46		Lots of *Pultenaea* + some *Burchardia*
47	F	~1:1 *Bossiaea* + *Pultenaea*
48	F	Lots of *Bossiaea heterophylla* pollen
49	F	Mix of *Pultenaea* + 2 others
50	F	Mostly *Pultenaea* + a few *Bossiaea* + some *Xanthorrhea*?
51	F	Lots of *Bossiaea obcordata* pollen + *Pultenaea* + some other
52	F	Lots of *Pultenaea* + some *Stylidium* + *Burchardia* + other
53	F	Lots of *Pultenaea* plus some *Bossiaea*
54	M	~ 8 *Burchardia* only
55	M	No pollen
56	F	Mostly *Stylidium* + 2 others
57	F	Mostly *Stylidium* + some *Pultenaea*
58	F	Mostly *Pultenaea* + *Stylidium*
59	F	Presumably *Xanthorrhea* pollen only (no ref.)
60	F	Mostly *Hardenbergia* + some *Pultenaea* + others
61	F	No pollen
62	F	Mostly *Pultenaea* + a few *Hardenbergia*
63	F	*Stylidium* pollen
64	M	No pollen
66	M	Mostly *Dillwynia* + 1 *Xanthorrhea* + some others
67	F	9 *Dillwynia* + 1 *Stylidium*
69	F	*Dillwynia* + orchid pollen
70	M	3 *Dillwynia* grains
71	M	Mostly *Dillwynia* pollen

73	F	Mostly *Dillwynia*
74	F worker	Some small unidentified grains
75	F worker	Lots of orchid pollen
76	F	Lots of *Dillwynia* pollen + 2 unidentified
77	F	A few unidentified grains
78	M	Some *Dillwynia* only
79	F	23 *Dillwynia* + 6 other
80	F	*Dillwynia* pollen only
81	F worker	No pollen
82	F	A few *Dillwynia* grains
83	F worker	No pollen
84	F	~30 *Dillwynia* grains
85	F	Mostly *Dillwynia* + some smaller grains
86	F	A few *Dillwynia* grains + some *Stylidium*
87	F worker	No pollen
88	F worker	No pollen
89	M	Some *Dillwynia* only
90	M	A few *Dillwynia* grains + 1 *Stylidium*
91	M	
92	M	A few *Dillwynia* grains
93	M	Mostly *Dillwynia* + some unidentified
94	F	A few *Dillwynia*
95	F	Mostly *Dillwynia* + a few *Stylidium*
96	F	Mostly *Dillwynia* + 2 others

97	M	No pollen

Table A1.3 (Cont'd)

Bee #	Comments (bee ID)	Comments (field)
1	chars consistent with *T. venustus*	
2		
3	genitalia and hidden sterna checked	
4		with orchid pollinarium
8		
9		
10		small native bee
11		small native bee
12	chars consistent with *T. venustus*	
13		
14		small native bee
15		with pollinarium remnant = *D. praecox* (AFLP)
17		
18		small bee with dancing flight
19		with orchid pollinarium = *D. alba* (AFLP)
20		
21		
22		with orchid pollinarium = *D. alba* (AFLP)
23	*Eurys* sp. (sawfly)	with pollinarium remnants

24		
26	this Eumenid wasp preys on moth larvae	same as previously on *D. praecox*?
27		native bee
28		to #35 near Anna Bay sign Nelson Bay Rd
29		
30		probably sun/shade plant habit forms
31		
32		
33		
34		
35		
36		under power lines near *D. arenaria*
37	chars consistent with *T. venustus*	
38	chars consistent with *T. venustus*	
39		
40		
41		
42		
43		
44		
45		
46		
47		native bee
48		"

49		"
50		"
51		
52	unnamed species JK221. Would like in AM collection	
53	may be synonymous with *L. phanerura*	
54		
55	I have found this only at Castlereagh NR. I hope it is the male of JK221	
56		
57	unnamed species JK221. Would like in AM collection	
58	unnamed species JK221. Would like in AM collection	
59		two tiny native bees
60	may be synonymous with *L. phanerura*	
61		very small native bee
62	may be synonymous with *L. phanerura*	
63	may be synonymous with *L. phanerura*	
64	I have found this only at Castlereagh NR. I hope it is the male of JK221	
66		
67		
69	probably *L. submoerens*	with orchid pollinarium = *D.* sp. aff. *punctata* (AFLP)
70		
71		
73	This group of *Euhesma* is still being revised. This specimen is near *E. palpalis*.	
74		very small bee species frequenting *Diuris*

75		
76		
77		
78		
79		
80	This group of *Euhesma* is still being revised. This specimen is near *E. palpalis*.	
81		lots of these small bees on *Diuris*
82		
83		
84		
85		
86		
87		common small bee
88		" " "
89		
90		
91		
92		
93	ID is tentative as genitalia are not quite as published	small native bee
94		near patch of *D. sulphurea*
95		three bees caught together
96		medium-sized bee
97		

Appendix 2

Diuris species and natural hybrids recognized as at June, 2008.

Diuris species list compiled by Mark Clements *:* 'Please see print co|'Pl
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. Classification scheme at genus, subgenus and section levels indicated in purple text with first publication source indicated.

Diuris Sm., Trans. Linn. Soc. London 4: 222 (1798). Type species: *Diuris aurea* Sm.

subgen. ***Diuris***

Sect. ***Diuris***

Synonym: *Diuris* sect. *Flaviflorae* G.Don in Loudon's Hortus Britannicus 368 (1830). Type species: *Diuris aurea* Sm., *fide* Jones and Clements (2006).

Diuris aurea Smith, Exotic Bot. 1: 15, t. 9 (1805).

Diuris byronensis **D.L.Jones, Orchadian 14(3): 132-133, f. 1, t. (2003).**

Diuris chrysantha D.L.Jones et M.A.Clem., Proc. Roy. Soc. Queensland 98: 130-2, f. 7 (1987).

Diuris disposita D.L.Jones, Austral. Orch. Res. 2: 55, f. 69 (1991).

Diuris flavescens D.L.Jones, Austral. Orch. Res. 2: 56, f. 71 (1991).

Diuris luteola D.L.Jones et B.Gray, Austral. Orch. Res. 2: 57-58, f. 73 (1991).

Diuris unica D.L.Jones, Austral. Orchid. Res. 5: 82, f.3.13, t. (21 Dec. 2006).

Diuris secundiflora Fitzg., Austral. orch. 1(4): [t. 9] (1878).

Diuris tricolor Fitzg., J. Bot. 23: 137 (1885).

sect. ***Purpureo-albae*** G.Don in Loudon's Hortus Britannicus 368 (1830). Type species: *Diuris alba* R.Br., *fide* Jones and Clements (2006).

Diuris alba R.Br., Prod. 326 (1810).

Diuris arenaria D.L.Jones, Orchadian 12(12): 567-568, f. 1, t. (1999).

Diuris callitrophila D.L.Jones, Orchadian 14(3): 133-135 (2003).

Diuris curta D.L.Jones, Austral. Orchid. Res. 5: 76-77, f. 3.9 (21 Dec. 2006).

Diuris daltonii (C.Walter) D.L.Jones et M.A.Clem., Orchadian 14(8): Sci. Suppl. xiv (June 2004).

Diuris dendrobioides Fitzg., Austral. orch. 1(7): [t. 3] (1882).

Diuris fragrantissima D.L.Jones et M.A.Clem., Austral. Orch. Res. 1: 68 (1989).

Diuris minor (Benth.) D.L.Jones et M.A.Clem., Orchadian 14(8): Sci. Suppl. xiv (June 2004).

Diuris oporina D.L.Jones, Austral. Orch. Res. 2: 59-60, f. 77 (1991).

Diuris parvipetala (Dockrill) D.L.Jones et M.A.Clem., Proc. Roy. Soc. Queensland 98: 132 (1987).

Diuris punctata Sm. var. ***punctata*** Exotic Bot. 1: 13, t. 8 (1804).

Diuris punctata Sm. var. ***sulfurea*** Rupp, Proc. Linn. Soc. New South Wales 69: 73 (1944).

subgen. ***Xanthodiuris*** D.L.Jones et M.A.Clem., Orchadian 15(5): 203 (2006). Type species: *Diuris maculata* Sm.

sect. ***Xanthodiuris***

Diuris aequalis F.Muell. ex Fitzg., Austral. orch. 1(2): [t. 6] (1876).

Diuris bracteata Fitzg., Austral. orch. 2(4): [t. 2] (1889).

Diuris cuneilabris Rupp, Proc. Linn. Soc. New South Wales 73: 134, f. 1 (1948).

Diuris goonooensis Rupp, Victorian Naturalist 72: 110 (1955).

Diuris maculata Sm., Exotic Bot. 1: 57, t. 30 (1804-05).

Diuris nigromontana D.L.Jones, Orchadian 15(12): 550-551, t. (June 2008).

Diuris pardina Lindl., Gen. sp. orchid. pl. 507 (1840).

Diuris platichila Fitzg., Austral. orch. 2(4): [t. 3] (1891).

Diuris semilunulata Messmer in Rupp, Orch. New South Wales 139-140 (1943).

sect. ***Abbreviatae*** D.L.Jones et M.A.Clem., Orchadian 15(5): 203-204 (2006).Type species: *Diuris* ***abbreviata*** Benth.

Diuris abbreviata Benth., Fl. Austral. 6: 329 (1873).

Diuris exitela D.L.Jones, Austral. Orch. Res. 2: 55-56, f. 70 (1991).

Diuris praecox D.L.Jones, Austral. Orch. Res. 2: 60, f. 78 (1991).

sect. ***Pedunculatae*** D.L.Jones et M.A.Clem., Orchadian 15(5): 204 (2006).Type species: *Diuris* ***pedunculata*** R.Br.

Diuris basaltica D.L.Jones, Austral. Orchid. Res. 5: 75-76, f.3.9 (21 Dec. 2006).

Diuris behrii Schldl., Linnaea 20: 572 (1849).

Diuris chryseopsis D.L.Jones, Austral. Orch. Res. 3: 74-75, f.4.1 (1998).

Diuris eborensis D.L.Jones, Austral. Orchid. Res. 5: 77-78, f.3.10, t. (21 Dec. 2006).

Diuris fucosa D.L.Jones, Austral. Orchid. Res. 5: 78, f.3.11, t. (21 Dec. 2006).

Diuris gregaria D.L.Jones, Austral. Orchid. Res. 5: 79-80, f.3.12 (21 Dec. 2006).

Diuris lanceolata Lindl., Gen. sp. orchid. pl. 508 (1840).

Diuris monticola D.L.Jones, Austral. Orch. Res. 3: 76-77, f.4.3 (1998).

Diuris ochroma D.L.Jones, Muelleria 8(2): 182-184, f. 2 d-f (1994).

Diuris pedunculata R.Br., Prod. 316 (1810).

Diuris protena D.L.Jones, Austral. Orchid. Res. 5: 81-82 (21 Dec. 2006).

Diuris subalpina D.L.Jones, Orchadian 15(12): 551, t. (June 2008).

Diuris venosa Rupp, Proc. Linn. Soc. New South Wales 51: 313, f. 1-6 (1926).

subgen. ***Hesperodiuris*** D.L.Jones et M.A.Clem., Orchadian 15(5): 204 (2006). Type species: *Diuris laxiflora* Lindl.

sect. ***Hesperodiuris***

Synonym: *Diuris* subgen. *Hesperodiuris* sect. *Setaceae* D.L.Jones et M.A.Clem., Orchadian 15(5): 204 (2006). Type species: *Diuris setacea* R.Br.

Diuris brevifolia R.S.Rogers, Trans. & Proc. Roy. Soc. South Australia 46: 148 (1922).

Diuris carinata Lindl., Gen. sp. orchid. pl. 510 (Sep. 1840).

Diuris concinna D.L.Jones, Austral. Orch. Res. 2: 53-54, f. 67 (1991).

Diuris drummondii Lindl. in Edwards's, Bot. Reg. 1-23: Swan Riv. Append. li (1840).

Diuris emarginata R.Br., Prod. 316 (1810).

Diuris filifolia Lindl. in Edwards's, Bot. Reg.1-23: Swan Riv. Append. li (1840).

Diuris heberlei D.L.Jones, Austral. Orch. Res. 2: 56-576, f. 72 (1991).

Diuris immaculata D.L.Jones, Austral. Orchid. Res. 5: 80-81 (21 Dec. 2006).

Diuris laxiflora Lindl. in Edwards's, Bot. Reg. 1-23: Swan Riv. Append li (1840).*D.*

Diuris micrantha D.L.Jones, Austral. Orch. Res. 2: 58-59, f. 75 (1991).

Diuris pauciflora R.Br., Prod. 316 (1810).

Diuris picta J.Drummond in Hooker's, J. Bot. 5: 347 (1853).

Diuris setacea R.Br., Prod. 316 (1810).

sect. ***Pyrophilae*** D.L.Jones et M.A.Clem., Orchadian 15(5): 204 (2006). Type species: *Diuris laevis* Fitzg.

Diuris laevis Fitzg., Gard. Chron. (new ser.), 17: 495 (1882).

Diuris purdiei Diels, J. Proc. Mueller Bot. Soc. Western Australia_ 1(11): 79 (1903).

sect. ***Palustres*** D.L.Jones et M.A.Clem., Orchadian 15(5): 204-205 (2006). Type species: *Diuris palustris* Lindl.

Diuris palustris Lindl., Gen. sp. orchid. pl. 507 (1840).

sect. ***Suffusae*** D.L.Jones et M.A.Clem., Orchadian 15(5): 205 (2006). Type species: *Diuris longifolia* R.Br.

Diuris amplissima D.L.Jones, Austral. Orch. Res. 2: 53-54, f. 65 (1991).

Diuris brumalis D.L.Jones, Austral. Orch. Res. 2: 53, f. 66 (1991).

Diuris conspicillata D.L.Jones, Austral. Orch. Res. 2: 54 -55, f. 68 (1991).

Diuris corymbosa Lindl. in Edwards's, Bot. Reg. 1-23: Swan Riv. Append. li (1840).

Diuris longifolia R.Br., Prod. 316 (1810).

Diuris magnifica D.L.Jones, Austral. Orch. Res. 2: 58, f. 74 (1991).

Diuris orientis D.L.Jones, Austral. Orch. Res. 3: 77-78, f.4.4 (1998).

Diuris porrifolia Lindl. in Edwards's, Bot. Reg. 1-23: Swan. Riv. Append. li (1840).

Diuris pulchella D.L.Jones, Austral. Orch. Res. 2: 61, f. 79 (1991).

Diuris recurva D.L.Jones, Austral. Orch. Res. 2: 61-62, f. 80 (1991).

subgen. ***Paradiuris*** D.L.Jones et M.A.Clem., Orchadian 15(5): 205 (2006). Type species: *Diuris sulphurea* R.Br.

Diuris sulphurea R.Br., Prod. 316 (1810).

subgen. ***Timordiuris*** D.L.Jones et M.A.Clem., Orchadian 15(5): 205 (2006). Type species: *Diuris fryana* Ridl.

Diuris fryana Ridl. in H.O.Forbes, Nat. Wand. East. Archip. 519 (1885).

Natural Hybrids

Diuris* ×*fastidiosa R.S.Rogers, Trans. & Proc. Roy. Soc. South Australia 51: 6-7 (1927).

Diuris* ×*nebulosa D.L.Jones, Austral. Orch. Res. 2: 59, f. 76 (1991).

Diuris* ×*palachila R.S. Rogers, Trans. & Proc. Roy. Soc. South Australia 31: 209-210 (1907).

Diuris* ×*polymorpha Messmer in Rupp, Orchids New South Wales, Suppl. 142 (1943)